THE ANESTHETIC ISOFLURANE INFLUENCES BASELINE FIRING AND DISRUPTS CHEMOSENSITIVITY OF 5-HT AND GABA RAPHÉ NEURONS

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A

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Abstract

General anesthetics are widely used in clinical and scientific contexts, but their molecular mechanisms, and how these mechanisms give rise to the state of anesthesia, are poorly understood. We investigated the influence of the volatile anesthetic isoflurane on serotonin (5-hydroxytryptamine; 5-HT) and γ-aminobutyric acid (GABA) synthesizing raphé neurons. These cell types have been proposed as central chemoreceptors, cells that sense changes in arterial CO_2/pH and stimulate respiratory output to regain homeostasis. We tested the hypotheses that isoflurane inhibits 5-HT neuron baseline firing, enhances GABA neuron baseline firing, and disrupts the chemosensitivity of both neuron types. We performed extracellular recordings in the medullary raphé using the unanesthetized *in situ* perfused brainstem preparation. Subsets of neurons were labeled with biotinamide using the juxtacellular labeling method and immunohistochemically identified by neurotransmitter phenotype. Results indicated that isoflurane inhibited action potential discharge in 5-HT neurons. Isoflurane inhibited action potential discharge in a subset of CO_2-inhibited putative GABA neurons and enhanced action potential discharge in a different subset of these neurons. Isoflurane disrupted the chemosensitivity of both 5-HT and GABA neurons. Disruption of 5-HT and GABA neuron chemosensitivity by isoflurane may contribute to the blunted hypercapnic ventilatory response that is a secondary effect of general anesthesia.

Dedication

I dedicate this dissertation to my parents, who make every opportunity possible for me.

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Chapter 1

Introduction

1.1 Introduction to general anesthetics

General anesthesia was first publically demonstrated with diethyl ether in 1846, and soon after, many different anesthetics, including nitrous oxide, chloroform, ethylene, cyclopropane, trichloroethylene, and divinyl ether, were popularly used in clinical practice. Some of these anesthetics were later discontinued due to issues with toxicity, flammability, and compromising side effects, and new agents such as halothane, isoflurane, enflurane, sevoflurane, and desflurane were developed. The wide variety of anesthetics used today are classified into two major groups: 1) intravenous anesthetics, such as propofol, etomidate, and ketamine; and 2) inhalational anesthetics, which include volatile anesthetics such as isoflurane, enflurane, halothane, sevoflurane, and desflurane, and gaseous inhaled anesthetics, such as cyclopropane, nitrous oxide, and xenon (Evers et al., 2006). The present study will distinguish between these different types of anesthetics by referring to the two major classes as: 1) intravenous anesthetics; and 2) volatile or inhalational anesthetics. This introduction will primarily focus on volatile inhalational anesthetics, as the present study was conducted using the volatile anesthetic isoflurane.

General anesthetics depress the central nervous system to a sufficient degree to allow for surgical or other noxious procedures. This central nervous system depression, generally referred to as the state of anesthesia, can be described as a collection of changes in

behavior and perception, which include amnesia, immobility in response to noxious stimuli, attenuation of autonomic responses to noxious stimuli, analgesia, and hypnosis (Evers et al., 2006). The precise pattern of behavioral change is different for each anesthetic agent, which has made it difficult to define general anesthesia as a precise behavioral state. Clinically, general anesthesia is understood as the state in which perception and reflex motor responses to stimuli, particularly painful stimuli, are sufficiently inhibited to allow for noxious procedures.

In addition to producing the general state of anesthesia, all general anesthetics exert significant secondary effects on organ systems and suppress homeostatic reflexes, and as a result, these drugs have a low therapeutic index. Given their relatively narrow margin of safety, anesthetics must be administered with careful consideration of their secondary effects and pharmacokinetic properties. Furthermore, these agent-specific properties must be evaluated in the context of the patient's age, medical condition, medication use, and surgical procedure.

The key point of administering anesthetics is to reach and then maintain the appropriate level of anesthesia, as an excess of anesthesia can cause injury and may even prove fatal. Every anesthetic agent produces the cardinal clinical features of immobility, hypnosis, analgesia, and amnesia, but these clinical signs of general anesthesia can be unreliable in assessing the level of anesthesia because the degree and pattern of each effect is different for each anesthetic agent. This issue was addressed in 1965 when Eger et al. introduced

the concept of anesthetic equipotency, which defines the minimum alveolar concentration (MAC) of anesthetic preventing movement in response to surgical stimulation in 50 % of subjects (Eger et al., 1965). MAC values measuring the potency of inhalational anesthetics in terms of the response to noxious stimulation have been determined for all anesthetics that are used clinically (Evers et al., 2006). These MAC values allow for measurement of an endpoint representing the clinical goal of immobility. Additional MAC values have also been developed that measure different endpoints, such as MAC awake, which defines the concentration of anesthetic that prevents response to verbal commands in 50 % of subjects, as well as MAC for suppression of memory. In summary, MAC values afford the ability to measure anesthetic potency, which is of critical importance when administering anesthetics, particularly in light of the low therapeutic index and significant secondary effects of these drugs.

MAC values allow for measurement of endpoints representing clinical goals that are common to all anesthetics, but the pharmacokinetics that mediate the changes in behavior and perception necessary to reach those endpoints are specific to each anesthetic. The solubility properties of inhalational anesthetics are fundamental to their kinetics. Typically, solubility is represented by the blood/gas partition coefficient, which indicates the ratio of the concentration of the two phases, blood and gas, when partial pressures are in equilibrium. All inhalational anesthetics are selectively soluble in fatty tissues, and due to this lipid affinity, the solubility in tissue is different than the solubility in blood (Evers et al., 2006). The solubility in the blood and tissue contributes to the uptake, distribution,

and elimination of anesthetics. In humans, the uptake of anesthetics depends on the ratio between the alveolar and inspired concentration (FA/FI), which represents the equilibrium state between body tissues and inspired concentration (Torri, 2010). The rate of rise of FA/FI depends on numerous factors, such as the blood/gas partition coefficient, alveolar ventilation, cardiac output, blood flow, and tissue capacity. The elimination of anesthetics can be defined by the decrease in alveolar concentration relative to the alveolar concentration determined at the end of anesthesia (FA/FAo). The lower the anesthetic solubility, the shorter the recovery time (Torri, 2010). The kinetics of uptake and elimination are specific to each anesthetic agent, and these processes are important for clinicians to understand when administering different anesthetic drugs to ensure safe induction and recovery from anesthesia.

The present study used the volatile anesthetic isoflurane, which is commonly used in both clinical and scientific practice. Isoflurane is relatively difficult to synthesize and purify, so its development in 1965 followed that of its isomer, enflurane (Eger, 1981). Many of the physical properties of isoflurane and enflurane are similar, and both drugs are currently approved for use by the Food and Drug Administration. Isoflurane is a methyl ethyl ether compound that is highly halogenated with five flourines and one chlorine, which renders the compound nonflammable over clinically useful concentrations. Isoflurane potency, as measured by MAC-immobility, is 1.15 % in 100 % oxygen (Eger, 1981). The blood/gas partition coefficient of 1.4 is relatively low, which indicates that isoflurane is poorly soluble in blood. As with all inhalational anesthetics, the low blood

solubility of isoflurane is coupled with a greater affinity for lipid tissues. Despite the relatively high tissue solubility of isoflurane, the extremely low blood solubility allows for a faster elimination time compared to other potent inhaled anesthetics. This rapid elimination is a significant advantage of isoflurane because it allows mental function to return to normal and the circulatory, respiratory, and neuromuscular depression to quickly reverse, and it also decreases the possibility of liver or kidney toxicity. As with all general anesthetics, isoflurane does exert significant secondary effects on organ systems, including decreased systemic arterial blood pressure, suppressed ventilation, and depressed neuromuscular transmission.

1.2 Secondary effects of general anesthetics

In addition to their depressive effects on the central nervous system, all general anesthetics suppress homeostatic reflexes and exert secondary effects on organ systems. The predominate effect of inhalational anesthetics is a decrease in systemic arterial blood pressure due to vasodilation, myocardial depression, suppressed baroreceptor control, and a general decrease in sympathetic tone (Evers et al., 2006). The magnitude of this response is variable among different anesthetics, but all cause hemodynamic effects to some degree, and this secondary effect is crucial to consider in the context of the surgical procedure and patient's medical condition, particularly for trauma victims who already have severely depleted intravascular volume. Hypothermia is another critical secondary effect of anesthesia, in which patients develop a colder body temperature (<36 °C) during surgery (Evers et al., 2006). All anesthetics lower the core temperature point at which

thermoregulatory vasoconstriction is activated, which compromises the body's normal defense against heat loss and causes hypothermia. Also, the metabolic rate decreases during anesthesia, which reduces heat generation and contributes to the hypothermic condition. Postoperative side effects, such as nausea and vomiting, continue to be problematic side effects of general anesthetics during the recovery period.

Respiratory depression is a particularly important point of concern during general anesthesia. Virtually all anesthetics cause significant changes in the control of breathing, which involves both chemical and behavioral control systems. Chemical control of breathing depends on the chemical composition of arterial blood (pH, PO_2, PCO_2) or interstitial fluid in the brainstem (pH, PCO_2), whereas behavioral control adjusts breathing in response to changes in state such as sleep, arousal, speech, exercise, and pain (Dahan and Teppema, 2003). Inhalational anesthetics, particularly volatile halogenated agents such as halothane and isoflurane, affect both chemical and behavioral control systems, and thus, they exert some of the most drastic effects on respiration.

All inhalational agents depress ventilation by reducing tidal volume, and the accompanying increase in respiratory rate does not compensate for reduced alveolar ventilation because it primarily results in increased dead space ventilation (Torri, 2010). This reduced alveolar ventilation causes $PaCO_2$ to increase. Under normal conditions, the change in the chemical composition of the blood and interstitial fluid would trigger a ventilatory response, but inhalational anesthetics disrupt the normal homeostatic response

to hypercapnic (increased CO_2) and/or hypoxic (decreased O_2) conditions. This blunted ventilatory response to hypercapnia and hypoxia involves anesthetic action at peripheral sites of respiratory control in the carotid bodies and central respiratory control networks in the central nervous system. Furthermore, inhalational anesthetics reduce the reflexes that maintain airways, including gag and cough reflexes (Evers et al., 2006). Due to these serious secondary effects, ventilation generally must be controlled or assisted during surgery to prevent apnea or periodic breathing.

1.3 Theories of general anesthetic mechanisms of action

Although general anesthetics are widely used in medical practice and scientific contexts, the mechanism of action of anesthetics is poorly understood. Anesthesia is used to block perception of and motor reflex responses to painful stimuli, and since the functional unit of perception and motor reflexes are neurons, it has long been assumed that anesthetics act by modulating neuronal activity. Numerous theories have been proposed to describe the mechanisms by which anesthetics modulate neural activity. In the mid 1800s, chloroform, ether, and nitrous oxide, three structurally diverse inhalational anesthetics, were widely used to produce the state of anesthesia. The fact that structurally unrelated compounds could produce the same behavioral effects of immobility, hypnosis, analgesia, and amnesia led to the first theory of anesthetic action, termed the unitary theory. Proposed by Claude Bernard in the 1870s, the unitary theory of anesthetic action postulated that anesthetics acted non-specifically through a common mechanism in the central nervous system (Leake, 1971). Approximately 30 years later, Meyer and Overton

observed a strong correlation between anesthetic potency and solubility in olive oil, and they supplemented the unitary theory with the Meyer-Overton rule, which proposed that volatile anesthetics act by nonspecifically perturbing lipid cell membranes (Miller, 1969). The unitary theory and Meyer-Overton rule dominated general thinking until the late 20th century, when the consensus began to shift toward the concept of anesthetics acting on specific protein targets (Franks and Lieb, 1978; Franks and Lieb, 1984). The current literature supports an agent-specific, multiple-mechanism theory of anesthetic action, in which anesthetics act on multiple molecular targets in different anatomical sites to produce the complex changes in behavior and perception that characterize the state of anesthesia (Campagna et al., 2003; Grasshoff et al., 2005; Torri, 2010).

Currently, ion channels are considered the most likely target of anesthetics (Campagna et al., 2003; Rudolph and Antkowiak, 2004). Ion channels mediate action potential conductance, resting membrane potential, and neurotransmitter release, all of which are fundamental aspects of neural activity and signaling; thus, these channels should be central to changes in behavior and consciousness, such as those observed in the state of anesthesia (Mashour et al., 2005). The field is now focused on relating molecular actions of anesthetics, most notably on ion channels, to behavioral states of anesthesia.

The majority of research on molecular mechanisms has focused on postsynaptic anesthetic action on the chloride channels gated by inhibitory $GABA_A$ receptors [receptor subtype for the inhibitory neurotransmitter γ-aminobutyric acid (GABA)]. These $GABA_A$

receptors are sensitive at clinical concentrations to a wide array of different anesthetics, including both intravenous and inhalational agents. It has been widely shown that anesthetics potentiate inhibitory currents through $GABA_A$ receptors and increase the sensitivity of these receptors to GABA, which inhibits postsynaptic neuronal excitability (Evers et al., 2006). The mechanism is proposed to involve direct binding of anesthetics to specific binding sites on $GABA_A$ receptors, since point mutations have been shown to eliminate anesthetic effects on the associated ion channels (Rudolph and Antkowiak, 2004), but interestingly, none of the general anesthetics compete with GABA for its binding site. The only anesthetics that do not affect $GABA_A$ receptors are ketamine, nitrous oxide, cyclopropane, and xenon, which instead inhibit the *N*-methyl-*D*-aspartate (NMDA) receptor that mediates excitatory glutamatergic neurotransmission (Evers et al., 2006). Anesthetic action on $GABA_A$ receptors has long been considered to play the primary role in producing the overall central nervous system depression characteristic of general anesthesia.

Despite the long-standing predominance of research on postsynaptic anesthetic action, a growing body of literature describes presynaptic sites of anesthetic action as critical components of anesthetic mechanisms (Griffiths and Norman, 1993; Pocock and Richards, 1993). Presynaptic sites are primarily implicated by studies indicating that anesthetics affect neurotransmitter release (Evers et al., 2006). Neurotransmitter release can be inhibited by direct anesthetic interaction with the neurotransmitter release machinery (Xie et al., 2013), or by inhibition of the action potential leading to

neurotransmitter release. For example, anesthetic action on sodium (Na^+) channels has been shown to inhibit action potential dependent neurotransmitter release (Hemmings, 2009; Herold and Hemmings, 2012). Additionally, anesthetics binding to two-pore domain potassium (K^+) channels can inhibit neurotransmitter release by hyperpolarizing neurons (Patel et al., 1999), which leads to decreased action potential dependent calcium (Ca^{2+}) influx and neurotransmitter release.

1.4 Working hypothesis describing central depression in anesthesia

The overarching effect of anesthetics is depressed central nervous system activity. Within the widely accepted agent-specific, multiple-mechanism theory of anesthetic action, the governing hypothesis is that the central depression results from enhanced inhibitory activity and depressed excitatory activity. There are multiple ways to achieve both enhanced inhibitory and depressed excitatory transmission. For example, inhibition may be enhanced by increasing the release of inhibitory neurotransmitters, potentiating the response of postsynaptic receptors, or by augmenting postsynaptic neuronal excitability by direct modulation of the membrane potential. Excitation may be depressed by blocking action potential propagation, enhancing presynaptic inhibition, or inhibiting the response mediated by postsynaptic receptors. The enhanced inhibitory transmission is thought to primarily involve anesthetic action on $GABA_A$ and glycine receptors, and the depressed excitatory transmission has been shown to involve action on glutamate, nicotinic acetylcholine, and serotonin receptors (Franks and Lieb, 1994; Campagna et al., 2003; Torri, 2010). As stated, a growing number of studies suggest that presynaptic

modulation of neurotransmitter release by anesthetics may also contribute to the enhanced inhibitory and depressed excitatory neurotransmission that is hypothesized to cause overall central nervous system depression. Mechanisms of anesthetic action have been explored in virtually all areas of the brain, and all areas exhibit this pattern of enhanced inhibitory activity and depressed excitatory activity for a net result of central nervous system depression during anesthesia.

1.5 Target populations in the medullary raphé of the brainstem

The research presented in this thesis focuses on the influence of the volatile anesthetic isoflurane on neurons in the medullary raphé region of the rat brainstem. We targeted neurons of two distinct phenotypes: 1) serotonin (5-hydroxytryptamine; 5-HT) synthesizing neurons and 2) γ-aminobutyric acid (GABA) synthesizing neurons. These two cell types are proposed central respiratory chemoreceptors, which are neurons that sense changes in arterial CO_2/pH and respond by stimulating the respiratory network responses that are necessary to regain homeostasis. Carbon dioxide is soluble in the blood, and thus, CO_2 levels are linked to pH through the equation $CO_2 + H_2O \rightarrow H^+ + CO_3^-$ (Richerson, 2004). To give a broad overview of central chemoreception, under conditions of increased CO_2, the pH of the blood decreases. Respiratory chemoreceptors in the brain sense this condition of hypercapnia/acidosis and stimulate respiratory networks to signal an increase in respiratory output. This neural signaling results in increased breath frequency and amplitude, which allows the organism to offload the excess CO_2 and return the pH of the blood to homeostatic conditions. The margin of

change in blood pH, as governed by the amount of blood protons (H^+), that can be tolerated is extremely small; an increase in blood protons as little as 0.1 μM is fatal (Richerson, 2004). Thus, central respiratory chemoreceptors that monitor arterial blood CO_2/pH and stimulate respiratory output are critical to survival.

Both serotonergic and GABAergic systems have been implicated in facilitation of the homeostatic response to hypercapnia/acidosis (Richerson, 2004). CO_2-stimulated 5-HT and CO_2-inhibited GABA neurons have been shown to be intrinsically chemosensitive in the medullary raphé *in vitro* (Richerson et al., 2001). Research using the unanesthetized rat *in situ* perfused decerebrate brainstem preparations suggests these raphé neurons also function as central chemosensors in the intact animal (Corcoran et al., 2008; Iceman et al., 2010; Corcoran et al., 2013; Iceman et al., 2013). We propose the "push – pull" model of central chemosensitivity, in which 5-HT and GABA raphé neurons provide excitatory and inhibitory drive, respectively, to the respiratory central pattern generator, which represents a network of neurons that control the rate and relative timing of respiratory muscle contraction, and to motor neuron pools, which control breath amplitude (Fig. 1.1). Hypercapnia has been shown to stimulate the excitatory serotonergic pathway and inhibit the inhibitory GABAergic pathway *in vitro* and *in situ* (Richerson et al., 2001; Corcoran et al., 2008; Iceman et al., 2010; Corcoran et al., 2013; Iceman et al., 2013). By the "push – pull" model, hypercapnic conditions cause 5-HT neurons to stimulate respiratory output directly by increasing excitatory drive to the respiratory central pattern generator and motor neuron pools, and GABA neurons to

stimulate respiratory output through disinhibition by decreasing inhibitory drive to the respiratory control network.

Studying the effect of isoflurane on both 5-HT and GABA neurons allowed us to examine how anesthetics effect cells involved in excitatory and inhibitory neurotransmission, respectively, which allowed us to place our results in context of the working hypothesis that central depression results in enhanced inhibitory and depressed excitatory activity. Targeting these specific neuron types also allowed us to observe the effect of isoflurane on neurons that are known to be involved in respiratory control networks and to study how isoflurane affects the chemosensitive properties of these neurons. Investigating the influence of isoflurane on neurons involved in central chemosensitivity may offer insight into the mechanisms underlying the blunted ventilatory response that is a well-known secondary effect of general anesthesia (Evers et al., 2006). The remainder of this introduction will provide general background on the two cell populations of 5-HT and GABA raphé neurons, discuss the specific aims and experimental approach for this research, and briefly review major findings.

1.6 Introduction to the 5-HT system

Central mechanisms for integrating stimuli and modulating control system responses are essential for maintaining homeostasis. The serotonergic system, an interconnected network of neurons that communicate via the amine neurotransmitter serotonin (5-hydroxytryptamine; 5-HT), has been proposed as one such central mechanism that

integrates and modulates a wide range of homeostatic functions. These serotonergic cell bodies are located in the midbrain and caudal regions and project to virtually all areas of the central nervous system, allowing for their critical involvement in maintaining homeostatic processes across the motor, somatosensory, and limbic systems (Jacobs and Azmitia, 1992; Lovick, 1997; Hornung, 2003; Kinney et al., 2009).

5-HT neurons are divided into two distinct groups based on their distinct anatomical locations, connectivity, and functions: 1) the rostral group of the midbrain and pons; and 2) the caudal group of the medulla (Fig. 1.2 adapted from Kinney et al., 2009). The rostral group (ascending serotonergic network), including the pontine raphé, dorsal raphé, and median raphé, project to the forebrain and mediate arousal, cognition, anxiety, and cerebral blood flow (Jacobs and Azmitia, 1992). These neural circuits have been implicated in numerous neuropsychiatric and neurological disorders. The caudal group (descending serotonergic network), including raphé obscurus, raphé pallidus, and raphé magnus in the midline and the parapyramidal region on the ventrolateral medullary surface (VLMS), project to the lower brainstem and spinal cord and mediate autonomic output, respiratory, cardiovascular, and motor control, and nociception (Richerson, 2004). The widespread involvement of both ascending and descending serotonergic networks in diverse neural circuits leads to their hallmark characteristic as integrators, modulators, and critical regulators of essential homeostatic processes.

1.7 Pathology associated with raphé 5-HT neurons

As serotonin is critical to the modulation and integration of diverse homeostatic functions, failure of the serotonergic system to function normally can have severe implications. Sudden Infant Death syndrome (SIDS) is defined as the unexpected death of an infant less than one year of age that is usually correlated with sleeping and cannot be explained after autopsy, death scene investigation, and review of clinical history. SIDS is thought to be due to a defective brainstem-mediated protective response to homeostatic stress during sleep in a critical period of life (Kinney et al., 2009). This failed protective response is described by a "Triple Risk Model," which involves three risk factors: 1) intrinsic factors that cause an underlying brainstem abnormality; 2) a critical developmental period during which rapid changes in cardiorespiratory control and sleep/wake cycles occur; and 3) exogenous stressors that cause hypoxia (decreased O_2), hypercapnia (increased CO_2), and asphyxia (combined hypoxia and hypercapnia), such as over-bundling and the prone sleep position (Fig. 1.3). Convergence of these three risk factors leads to the failed protective response, in which SIDS infants cannot adjust to lethal, asphyxia-producing circumstances that normal infants would survive (Kinney et al., 2009).

Pathologic findings identify an intrinsic risk factor for SIDS to be abnormalities in the development of the medullary serotonergic system (Panigrahy et al., 2000; Kinney et al., 2003; Paterson et al., 2006). Compared to controls, SIDS cases exhibit abnormal medullary 5-HT pathology characterized by decreased 5-HT_{1A} receptor-binding sites in

the medulla, increased 5-HT cell number and density, immature cell morphology, and reduced binding to the 5-HT transporter. These results are consistent with abnormal 5-HT firing, synthesis, release, and clearance (Paterson et al., 2006). An example of SIDS pathology resulting in increased 5-HT cell density and decreased 5-HT_{1A} receptor-binding sites is shown in Figure 1.4 (adapted from Paterson et al., 2006). Abnormalities in the 5-HT system may translate into unexpected infant death because the 5-HT system is required to maintain multiple homeostatic functions that are crucial to autoresuscitation and survival, including cardiorespiratory reflexes, temperature regulation, arousal, and breathing.

1.8 Role of medullary raphé 5-HT neurons in central respiratory control

Arguably the most critical homeostatic function mediated by 5-HT neurons that is relevant to SIDS pathology is cardiorespiratory homeostasis. Medullary raphé 5-HT neurons have been studied extensively for their predominant role in the control and modulation of breathing. A wide array of anatomic evidence indicates that 5-HT neurons are located in respiratory networks. Serotonin-immunoreactive nerve terminals, arising from 5-HT neurons in the medullary raphé and ventrolateral medulla, are found in all major respiratory nuclei, including the phrenic motor nucleus, hypoglossal nucleus, retrotrapezoid nucleus, pre-Bötzinger complex (pre-BötC), nucleus ambiguus, and nucleus tractus solitarius (Pilowsky et al., 1990; Voss et al., 1990; Jacobs and Azmitia, 1992; Ptak et al., 2009). These respiratory nuclei also contain nerve terminals that are immunoreactive for substance P (SP) and thyrotropin-releasing hormone (TRH),

neuropeptides that colocalize in medullary raphé 5-HT neurons (Holtman et al., 1984; Holtman, 1988). Receptors for raphé neurotransmitters and neuropeptides are also found present in the major respiratory nuclei (Richerson, 2004). Additionally, medullary raphé 5-HT neurons receive reciprocal projections from some major respiratory nuclei, such as the pre-BötC neurons responsible for inspiratory rhythm generation (Ptak et al., 2009). This anatomical evidence indicates that medullary raphé 5-HT neurons are embedded within the central respiratory network.

Given the anatomical evidence indicating that 5-HT neurons are embedded within respiratory networks, much research is aimed toward defining the functional role of 5-HT neurons in respiratory circuits. Activation of receptors in the major respiratory nuclei via exongenous application of 5-HT, SP, and TRH has been widely shown to stimulate breathing, both *in vitro* and *in vivo* (Richerson, 2004). The contribution of endogenous 5-HT and raphé neuropeptides to respiratory circuits, however, was a long-held source of controversy, with contradictory conclusions that 5-HT inhibits, excites, or has no effect on breathing (Hodges and Richerson, 2010), until recently, experiments from Ptak et al. (2009) provided clear evidence that endogenous 5-HT provides a necessary tonic, excitatory drive to respiratory circuits. These experiments in neonatal rat medullary slices *in vitro*, as well as in the arterially perfused brainstem – spinal cord preparations *in situ*, demonstrated that simultaneous release of 5-HT and SP, activating 5-$HT_{2A/2C}$, 5-HT_4, or NK-1 receptors, is required to maintain inspiratory motor output. The study showed that some raphé obscurus 5-HT neurons project to the pre-Bötzinger complex and hypoglossal

motor nucleus, and stimulating raphé obscurus activity results in increased motor output due to excitation of pre-BötC and motor neurons. The study also showed that 5-HT can cause some pre-BötC neurons to display intrinsic bursting activity, which suggests an additional role for 5-HT in rhythm generation. Additional studies in knockout mice indicated that genetic elimination of 5-HT neurons disrupts respiratory rhythm *in vivo* and leads to high postnatal mortality in neonates (Hodges et al., 2009). Collectively, these data allow for the conclusion that 5-HT neurons function within respiratory circuits to stimulate breathing.

1.9 Identity of 5-HT neurons as central chemoreceptors is controversial

Although it is clear that 5-HT neurons stimulate respiratory output, their identity as central chemoreceptors has not been conclusively demonstrated. As stated previously, central chemoreceptors are neurons that sense changes in arterial CO_2/pH and respond by stimulating the respiratory network responses that are necessary to regain homeostasis. Under normal circumstances, central chemoreceptors detect small changes (1 to 2 mmHg) in arterial PCO_2 and elicit changes in breathing frequency and amplitude to maintain blood PCO_2 at a steady state (40 mmHg). Under situations of stress, such as asphyxia-producing circumstances, central chemoreceptors detect abnormally high levels of arterial PCO_2 (on the order of tens of mmHg) and elicit relatively large changes in breathing (Guyenet et al., 2010).

To be conclusively identified as a central respiratory chemoreceptor, cells must meet two essential criteria: 1) express intrinsic chemosensitivity to physiologically relevant changes in arterial pH/CO_2; and 2) exert specific effects on respiration, as in, they must respond to an increase in CO_2 by increasing respiratory output (Richerson et al., 2005). Firstly, demonstrating that chemosensitivity is an intrinsic mechanism is practically difficult. The cell must be physically separated from all other cells to eliminate modulatory effects, but this approach may eliminate connections that are integral to the cell's ability to express intrinsic chemosensitivity. Pharmacological blockade of synaptic transmission is another method for cell isolation, but these methods are less complete, since the typical method of applying glutamate or GABA receptor antagonists to eliminate excitatory or inhibitory modulatory effects, respectively, does not block other forms of neurotransmission, and decreasing calcium does not block non-vesicular transmission. The difficulty of isolating a potential central respiratory chemoreceptor impedes conclusive determination of intrinsic chemosensitivity. Secondly, defining the mechanism by which a potential central respiratory chemoreceptor exerts specific effects on respiratory output has also proved difficult, since many different proteins, ion channels, and neurons are pH-sensitive to some degree. The cellular mechanisms underlying the vital homeostatic process of central chemoreception, and the identity of neurons that function as respiratory chemoreceptors, is still much debated (Guyenet et al., 2005; Richerson et al., 2005; Nattie and Li, 2009; Guyenet et al., 2013).

It is generally accepted that central chemoreception involves multiple different sites and mechanisms (Nattie and Li, 2009). The current literature proposes two main candidates as potential central respiratory chemoreceptors: glutamatergic neurons in the retrotrapezoid nucleus (RTN) (Mulkey et al., 2004; Guyenet et al., 2008; Guyenet and Mulkey, 2010) and serotonergic neurons in the medullary raphé (Richerson et al., 2001; Richerson, 2004; Hodges and Richerson, 2010; Corcoran et al., 2013; Iceman et al., 2013). Noradrenergic, orexinergic, and histaminergic neurons of the locus coeruleus, all of which have wake-promoting capabilities, have also been proposed as potential central chemoreceptors (Guyenet et al., 2010). Some research also supports a role of glial cells, specifically astrocytes, which exhibit pH-induced ATP release that stimulates the proposed chemoreceptive RTN neurons (Gourine et al., 2010).

Medullary raphé 5-HT neurons are proposed as potential central respiratory chemoreceptors. These cells do have some properties that are consistent with central chemoreceptors. In terms of anatomical location, 5-HT neurons are closely associated with the basal artery and its major branches, which is an ideal location for monitoring arterial blood pH/CO_2 (Fig. 1.5 adapted from Bradley et al., 2002). As previously discussed, there is a dense concentration of 5-HT immunoreactive nerve terminals in major respiratory nuclei, such as the pre-BötC and hypoglossal motor nucleus, and stimulating raphé obscurus activity results in increased motor output due to excitation of pre-BötC and motor neurons (Ptak et al., 2009). Although it is well established that 5-HT neurons are embedded in respiratory networks and function to stimulate breathing, their

identity as central chemoreceptors remains a source of controversy because of contradictory evidence regarding the response of 5-HT neurons to hypercapnia. Serotonergic neurons in the medullary raphé nuclei have been identified as chemosensitive *in vitro* and *in situ* (Richerson et al., 2001; Corcoran et al., 2013; Iceman et al., 2013). A representative serotonergic neuron that was stimulated by CO_2 *in vitro* is shown in Figure 1.6 (adapted from Wang et al., 2001). Although CO_2-stimulated 5-HT neurons are identified *in vitro* and *in situ*, some electrophysiological studies on anesthetized rats *in vivo* do not find 5-HT neurons to be chemosensitive. Mulkey et al. (2004) reported that 5-HT neurons in the VLMS do not respond to inhalation of 10 % CO_2 in halothane anesthetized rats *in vivo*. Corroborating these findings, DePuy et al. (2011) found that 5-HT neurons in raphé obscurus do not respond to inhalation of 10 % CO_2 in isoflurane anesthetized rats *in vivo*. However, *in vivo* studies on awake, unanesthetized, freely-moving cats found that a subset of 5-HT neurons in the medulla and midbrain increase firing in response to inhalation of as little as 3 % CO_2 (Veasey et al., 1995; Veasey et al., 1997). Due to these contradictory findings regarding the response of 5-HT neurons to hypercapnia *in vivo*, the identity of 5-HT neurons as central chemoreceptors has been a long-standing source of controversy.

Previously, we hypothesized that use of anesthetics during neuronal recording was the primary reason for the lack of chemosensitive raphé neuronal subtypes identified in anesthetized rat *in vivo* preparations. In collaboration with in culture and *in vivo* studies from the Richerson laboratory at the University of Iowa, we established that the

anesthetic isoflurane disrupts the chemosensitivity of 5-HT neurons *in situ* (Johansen et al., 2012; Massey et al., 2013). Our observations of the severe artifact introduced by anesthesia may explain why *in vivo* experiments on anesthetized rats do not find 5-HT neurons to be chemosensitive (Mulkey et al., 2004; DePuy et al., 2011). I contributed to the data collection and manuscript preparation for this project, and our manuscript has been submitted for publication and is included in the appendix of this thesis. Building on this study of how isoflurane affects the chemosensitivity of 5-HT neurons, we targeted how isoflurane affects action potential discharge in 5-HT neurons, and these data are presented in the second chapter of this thesis.

1.10 Role of medullary raphé GABA neurons in central respiratory control

Having established the effect of isoflurane on the basal activity and chemosensitivity of 5-HT neurons, the proposed excitatory chemosensors in our "push – pull" model, we then focused on defining how isoflurane affects GABA neurons, which are the inhibitory chemosensors in our model (Fig. 1.1). Studies *in vitro* have identified GABAergic neurons in the medullary raphé that are inhibited by hypercapnia (Wang et al., 1998; Zhang et al., 2003; Richerson et al., 2005). These neurons are proposed to disinhibit respiratory output during hypercapnia (Wang et al., 2001; Richerson, 2004), which supports the function of GABA neurons in central respiratory control that we proposed in our "push – pull" model. Research in the arterially perfused rat brainstem *in situ* preparation provides additional evidence for medullary raphé GABA neurons that function as central chemosensors (Corcoran et al., 2008; Iceman et al., 2010). Studies *in*

vivo also indicate that GABA mechanisms are involved in homeostatic responses to hypercapnia (Gourine and Spyer, 2001; Liu et al., 2003; Kuribayashi et al., 2008). Collectively, these studies serve as strong evidence that central chemosensitivity involves input from GABA neurons. Investigating how isoflurane affects the chemosensitivity of neurons that are known to contribute to central chemosensitivity may afford important insight into the neuronal mechanisms that give rise to the decreased ventilatory hypercapnic response that is a well-known secondary effect of general anesthesia.

In addition to the potential to contribute to our understanding of the mechanisms governing respiratory depression during anesthesia, studying the effect of isoflurane on action potential discharge in GABA neurons is an important research focus because although the GABA system has been a major focus of research on anesthetic mechanisms, the majority of research targets postsynaptic anesthetic action on $GABA_A$ receptors, and little is known about how anesthetics affect GABA neurons themselves. Select studies have targeted how anesthetics affect neurotransmitter release from GABA neurons, which represents presynaptic anesthetic action, but evidence on how anesthetics affect GABA release is controversial. Both inhibition and enhancement of neurotransmitter release of GABA in response to anesthetic has been reported. For example, in the CA1 region of rat hippocampal slices, general anesthetics were shown to cause an increase in inhibitory postsynaptic current (IPSC) frequency, which was attributed to a presynaptic mechanism (Pittson et al., 2004), but in a different study, volatile anesthetics were shown to decrease action potential dependent GABA release in

synaptic boutons isolated from rat hippocampal CA1 neurons (Ogawa et al., 2011). Our study monitoring action potential discharge, and by extension, action potential dependent neurotransmitter release, in medullary raphé CO_2-inhibited putative GABA neurons may lend insight into how anesthetics affect neurotransmitter release of GABA. These data will contribute to literature that describes presynaptic sites of anesthetic action.

As a general note, this thesis will typically refer to medullary raphé GABA neurons as "CO_2-inhibited putative GABA neurons." Unlike 5-HT neurons, which have a characteristic firing pattern and can by confirmed as 5-HT neurons by the Mason algorithm (Mason, 1997), medullary raphé GABA neurons display a wide variety of firing patterns, and there is not a mathematical algorithm that can identify a neuron as GABAergic. Thus, we rely on the juxtacellular labeling method in combination with immunohistochemistry to identify recorded neurons by neurotransmitter phenotype. Although we are limited by our inability to conclusively identify all CO_2-inhibited neurons as GABAergic, due to the fact that the juxtacellular labeling method is, by nature, a low-yield process, our identification of CO_2-inhibited cells as GABA-synthesizing neurons is supported by prior *in vitro* and *in situ* studies that document a population of GABA neurons that are inhibited by hypercapnia (Corcoran et al., 2008; Iceman et al., 2010). We do not propose that all GABA neurons in the medullary raphé are CO_2-inhibited, but we have yet to document a CO_2-inhibited neuron that was identified as a non-GABA neuron. Thus, we will typically refer to this population of neurons as CO_2-inhibited putative GABA neurons.

1.11 Specific aims

I aimed to identify how isoflurane affects the basal activity of 5-HT neurons in the medullary raphé. We tested the hypothesis that isoflurane inhibits baseline firing of medullary raphé 5-HT neurons *in situ*. Documenting how isoflurane affects 5-HT action potential discharge has three important applications: 1) to contribute to our understanding of anesthetic mechanisms involving 5-HT neurons; 2) lend important insight into interpretation of results from *in vivo* studies conducted in animals maintained under anesthesia and aid in future experimental design involving anesthesia; and 3) add to the growing body of literature describing presynaptic mechanisms of anesthetic action. This research is reported in the second chapter in this thesis.

I also aimed to identify how isoflurane affects the basal activity and chemosensitivity of CO_2-inhibited putative GABA neurons in the medullary raphé. We tested the hypothesis that isoflurane enhances action potential discharge of medullary raphé CO_2-inhibited GABA neurons and disrupts their chemosensitivity *in situ*. Investigating how anesthetics affect the action potential discharge and chemosensitivity of medullary raphé CO_2-inhibited putative GABA neurons has three important applications: 1) to contribute to our understanding of anesthetic mechanisms involving GABA neurons, which may lend insight into molecular mechanisms that give rise to the state of anesthesia; 2) add to the growing body of literature describing presynaptic mechanisms of anesthetic action, which is a particularly germane contribution to the field, as the vast majority of prior research focuses on postsynaptic anesthetic action on $GABA_A$ receptors; and 3) offer insight into

the central mechanisms that contribute to the depressed ventilatory response to hypercapnia during anesthesia. This research is reported in the third chapter in this thesis.

1.12 Experimental approach

I addressed the following questions: 1) how does isoflurane affect the action potential discharge of medullary raphé 5-HT neurons *in situ*; and 2) how does isoflurane affect the action potential discharge and chemosensitivity of medullary raphé GABA neurons *in situ*. To record action potentials in 5-HT and GABA neurons, I performed extracellular recordings on single neurons in the rat brainstem and monitored how action potential discharge changed during isoflurane treatment. I labeled a subset of these neurons with biotinamide using the juxtacellular labeling method (Pinault, 1996; Winkler et al., 2006). By this method, I applied a current stimulus that was ejected through the electrode until the cell was entrained to fire simultaneously with the applied stimulus. Injected current, ejection of biotinamide, and cellular firing entrainment caused uptake of the biotinamide marker by the recorded cell. Brainstems were then removed for further processing, and I performed immunohistochemistry to identify the labeled neuron by neurotransmitter phenotype.

For all experiments, I used the *in situ* brainstem preparation as originally described (Paton, 1996) and in general usage in our laboratory (Corcoran et al., 2013; Iceman et al., 2013). A simplified diagram of this preparation is shown in Figure 1.7. A complete description of the methods used to generate this preparation is provided in the appendix.

Briefly, the preparation is bisected sub-diaphragmatically, decerebrated rostral to the superior colliculi, and the descending aorta is isolated to allow for arterial perfusion with artificial cerebral spinal fluid. Following removal of the surrounding bone and musculature, the surface of the brainstem is exposed to allow for neuronal recording. This preparation demonstrates eupnea-like respiratory activity, which indicates that the medulla is adequately oxygenated to preserve the normal activity of brainstem respiratory neural networks (Paton, 1996). We have previously shown that this preparation allows for successful use of electrophysiological recording, juxtacellular labeling, and immunohistochemistry techniques (Iceman et al., 2013).

1.13 Major findings

Here we present the first evidence that isoflurane inhibits the baseline firing of caudal raphé 5-HT neurons in the descending 5-HT system. Isoflurane routinely inhibited the baseline firing of 5-HT neurons or caused firing to cease completely. These results were consistent for all tested concentrations of isoflurane (1, 1.5, and 2 %), and the degree of inhibition increased as the concentration of isoflurane in extracellular solution increased. We found a significant difference between neuronal responses to 1 and 2 % isoflurane, which indicated dose responsiveness of 5-HT neurons to isoflurane within a clinically relevant range. 1 % isoflurane approximated 1.15 MAC, the minimum alveolar concentration for isoflurane (Eger, 1981). This concentration was confirmed by gas chromatography – mass spectrometry analysis. This analysis allowed us to confirm that our results from experiments *in situ* are relevant to evaluating results from *in vivo*

experiments on anesthetized animals, which typically are anesthetized within the range of 1 to 2 % inhaled anesthetic. Furthermore, confirming that the concentration of isoflurane received by the preparation was equivalent to 1.15 MAC allowed us to ensure that our results documenting the influence of isoflurane on 5-HT neurons are clinically relevant.

Here we present the first evidence documenting heterogeneity in the response of medullary raphé CO_2-inhibited putative GABA neurons to isoflurane. We found that CO_2-inhibited cells respond to isoflurane in one of two distinct ways: action potential discharge of these cells either significantly decreased, to the point of ceasing in the majority of cases, or dramatically increased in response to 1 % isoflurane. Comparing the baseline firing characteristics of cells that were inhibited to cells that were stimulated by isoflurane, we found a significant difference in baseline firing rates, in which isoflurane-stimulated neurons fired slowly at baseline (mean firing frequency: 0.50 ± 0.38 Hz) compared to isoflurane-inhibited neurons (mean firing frequency: 2.72 ± 0.70 Hz). Our results indicate a heterogeneous population of medullary raphé CO_2-inhibited putative GABA neurons represented by at least two distinct groups, which differ in their response to isoflurane and baseline firing frequency: 1) "slow-firing" CO_2-inhibited putative GABA neurons that are stimulated by isoflurane; and 2) "fast-firing" CO_2-inhibited putative GABA neurons that are inhibited by isoflurane.

In terms of the influence of isoflurane on chemosensitivity, we found that isoflurane disrupts chemosensitivity in 5-HT neurons, and these data are presented in the attached

manuscript (Massey et al., 2013). Here, we show that isoflurane disrupts chemosensitivity in the population of "slow-firing" isoflurane-stimulated CO_2-inhibited putative GABA neurons. Chemosensitive responses could not be resolved in the population of "fast-firing" isoflurane-inhibited CO_2-inhibited putative GABA neurons because these neurons ceased firing with isoflurane. We observed that the chemosensitive properties of both CO_2-stimulated 5-HT and CO_2-inhibited GABA neurons in the medullary raphé were disrupted by isoflurane.

Collectively, this research will contribute to our understanding of how anesthetics affect action potential discharge in medullary raphé neurons, which may offer insight into mechanisms of general anesthesia. Documenting how anesthetics affect the chemosensitivity of medullary raphé neurons that are known to be involved in respiratory circuits may help define molecular mechanisms underlying the decreased ventilatory response to hypercapnia that is characteristic of general anesthesia. Furthermore, this research indicating that medullary raphé neurons are profoundly affected by anesthetics, both in terms of their baseline firing and chemosensitive properties, will aid in interpretation of results from *in vivo* studies on anesthetized rodents and may inform future design of experiments that require anesthesia for conducting neuronal recordings. Finally, these data will add to the growing body of literature describing presynaptic mechanisms of anesthetic action by documenting how action potential discharge in medullary raphé 5-HT and GABA neurons is affected by isoflurane.

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1.15 Figures

Continued on the following pages

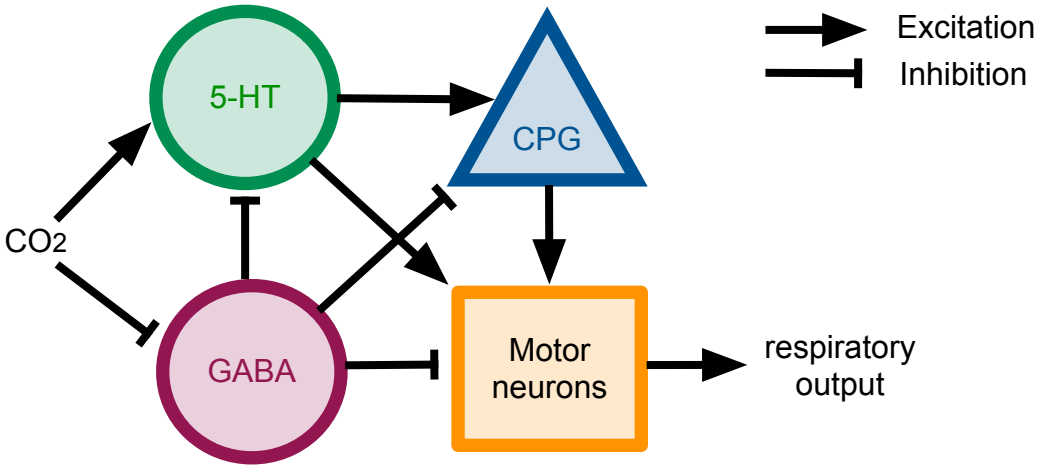

Figure 1.1. "Push - pull" model of central chemosensitivity. We propose the "push – pull" model of central chemosensitivity, in which raphé 5-HT and GABA neurons provide excitatory and inhibitory drive, respectively, to the central pattern generator (CPG), a network of neurons that control the rate and relative timing of respiratory muscle contraction, and to motor neuron pools, which control breath amplitude. By this model, increased CO_2 stimulates 5-HT neuron firing and release of the excitatory neurotransmitter serotonin, which stimulates activity in the CPG and motor neurons and leads to increased respiratory output. Increased CO_2 inhibits GABA neuron firing and release of the inhibitory neurotransmitter GABA, which decreases the inhibitory input to 5-HT neurons, the CPG, and motor neuron pools, leading to increased respiratory output through disinhibition.

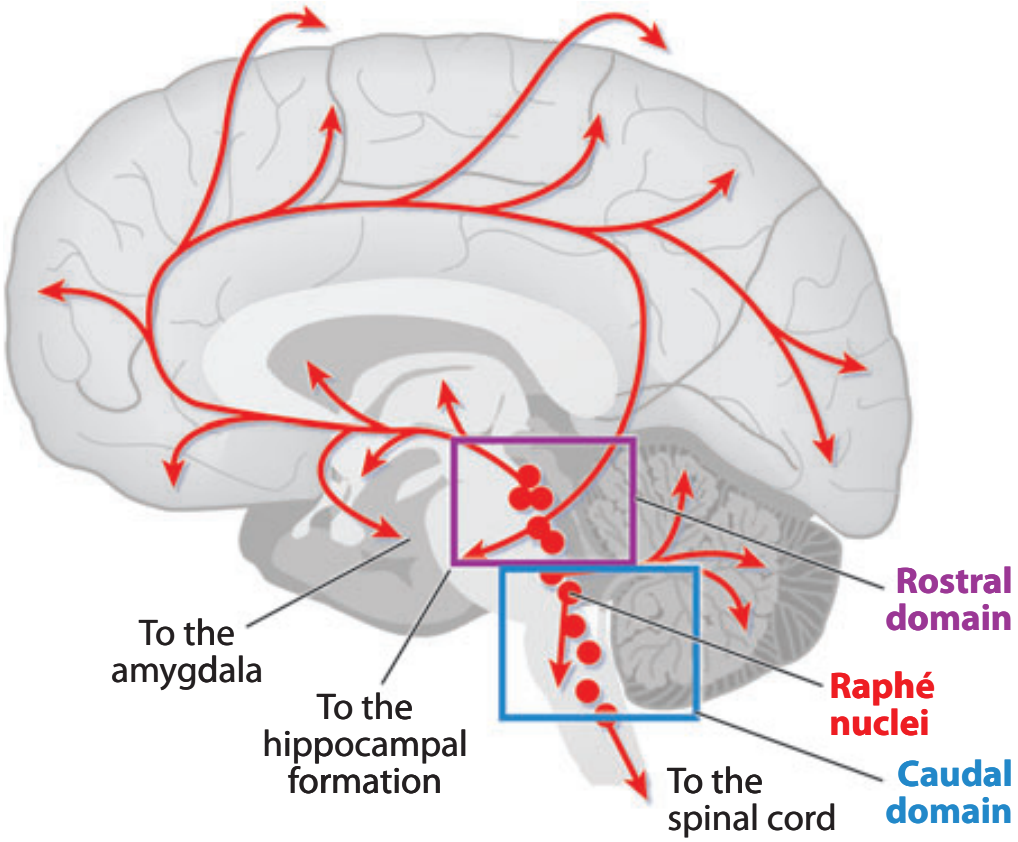

Figure 1.2. Serotonergic cell bodies and their projections (adapted from Kinney et al., 2009). Sagittal view of the human brain showing the sites of serotonergic cell bodies (red circles) and their projections (red lines). 5-HT neurons in the rostral domain are located in the rostral pons and midbrain, and these cells project to the cerebral cortex, thalamus, hypothalamus, hippocampus, and basal ganglia. 5-HT neurons in the caudal domain are located in the medulla. These cells project to other brainstem regions, the cerebellum, and the spinal cord.

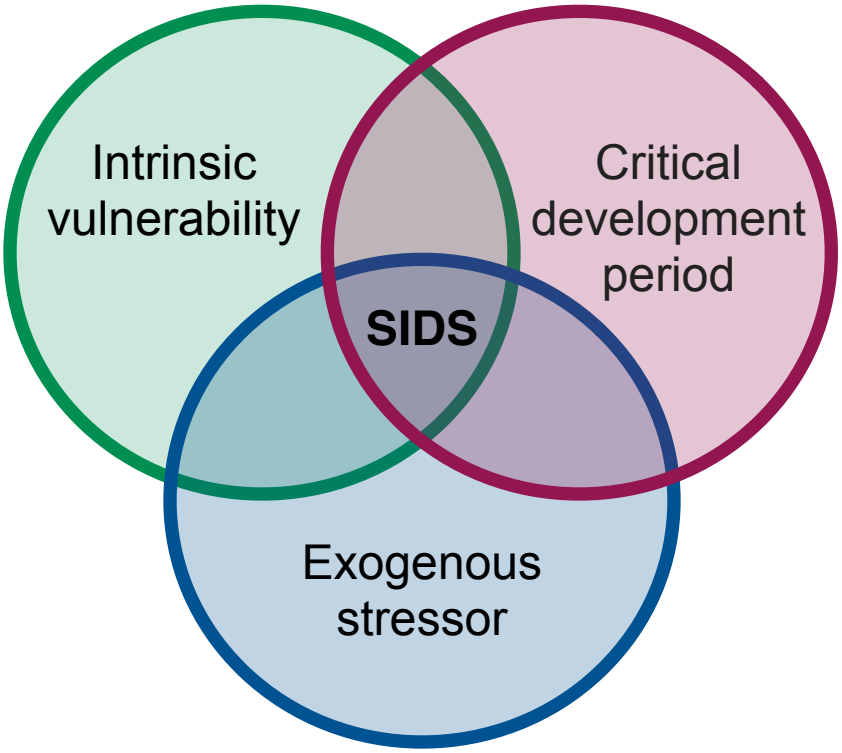

Figure 1.3. The "Triple Risk Model" describing three risk factors that are proposed to contribute to Sudden Infant Death Syndrome (SIDS). SIDS is thought to be due to a failed brainstem-mediated protective response to homeostatic stress during sleep in a critical period of life. The occurrence of SIDS is thought to involve the convergence of three risk factors: 1) intrinsic vulnerability caused by brainstem abnormality; 2) a critical development period when changes in cardiorespiratory control and sleep/wake cycles occur; and 3) exogenous stressors that cause hypoxia, hypercapnia, and asphyxia.

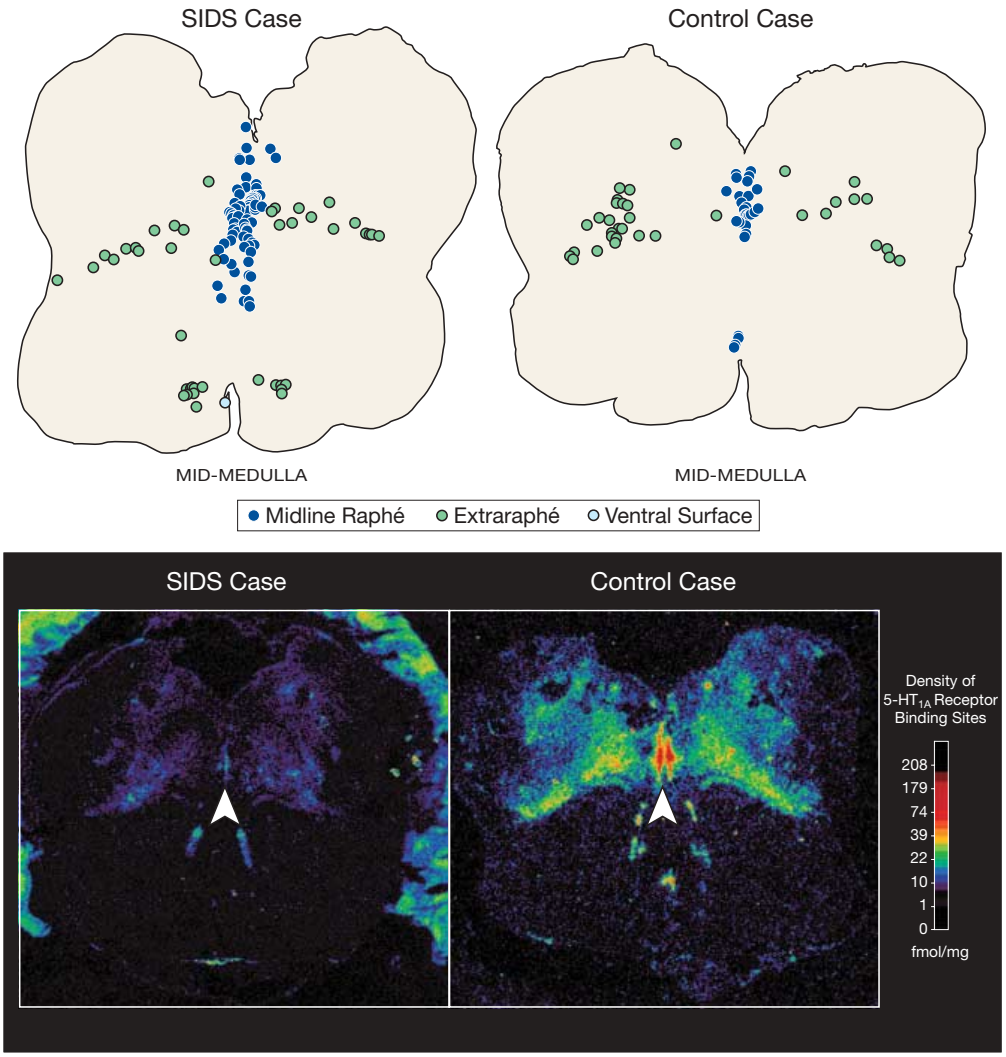

Figure 1.4. 5-HT neuron count and 5-HT1A receptor binding sites in a SIDS case compared to a control case (adapted from Paterson et al., 2006). Top panel: Distribution of 5-HT neurons in an infant dying from Sudden Infant Death Syndrome (SIDS case) and an infant with acute death from a non-SIDS cause (control case). The SIDS case has an increased number of 5-HT neurons, particularly in the midline, compared to the control. Bottom panel: Autoradiogram indicating mean 3H-8-OH-DPAT (labeled probe) binding to 5-HT1A receptors in a mid-medulla tissue section from an infant dying from SIDS and an infant with acute death from a non-SIDS cause. Density of 5-HT1A receptor binding sites, particularly in raphé obscurus (arrowheads), is lower in the SIDS case compared to the control.

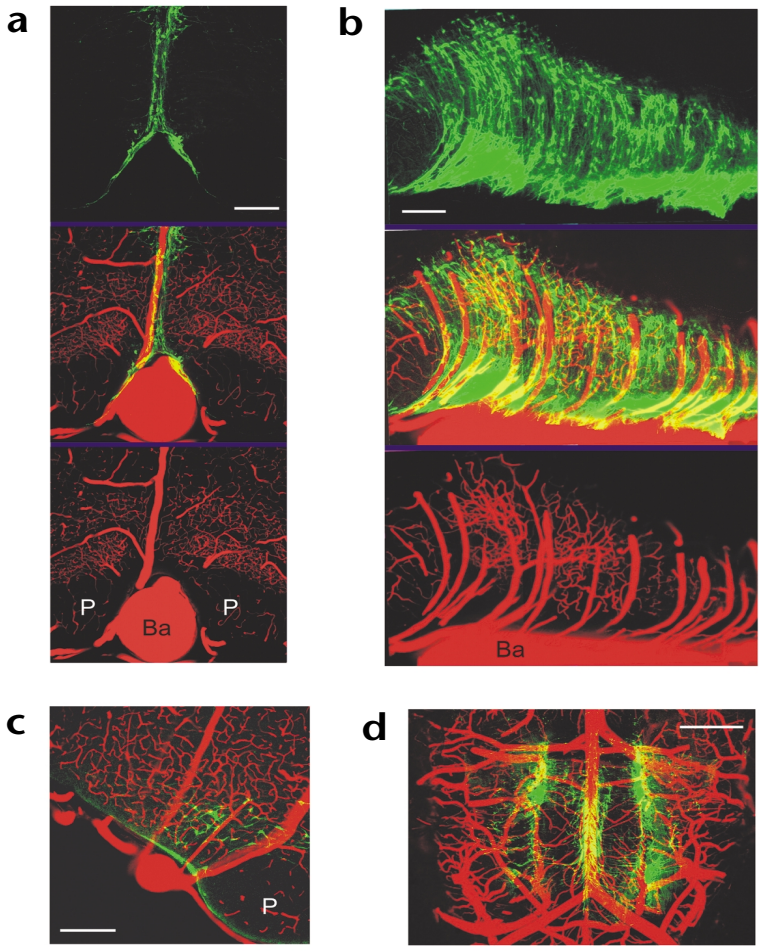

Figure 1.5. 5-HT neurons are closely associated with major arteries in the medullary raphé (adapted from Bradley et al., 2002). Confocal images of arteries filled with fluorescein-tagged albumin (red) and serotonergic neurons stained with anti-tryptophan hydroxylase (anti-TpOH) antibody (green). Colocalization of fluorescent markers (yellow) indicates that 5-HT neurons are highly concentrated along major arteries. **a)** Transverse section showing TpOH-immunoreactive neurons (top panel), arteries (bottom panel), and colocalization (middle panel). **b)** Mid-sagittal section (rostral side at right). **c)** Transverse section of the rostral ventrolateral medulla. **d)** Ventral surface of the medulla (filled vessels include arteries and some veins). Scale bars = 200 µm in **(a-c)** and 1.5 mm in **(d)**. Ba, basilar artery; P, pyramidal tracts.

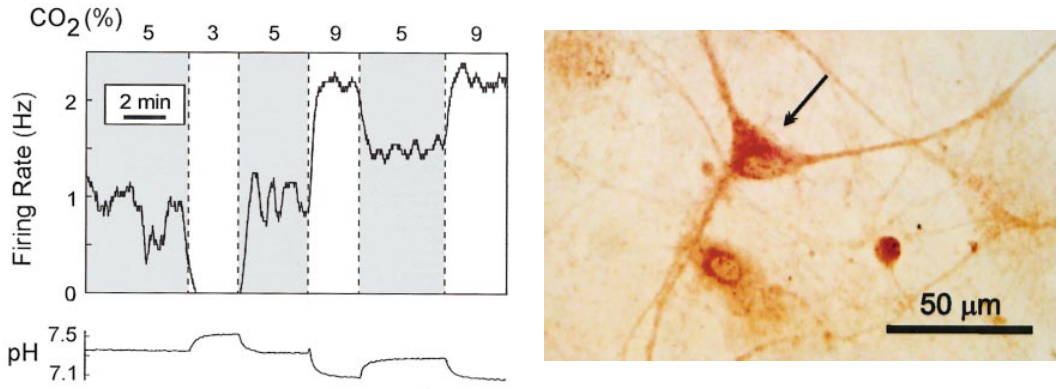

Figure 1.6. Acidosis-stimulated neurons in the medullary raphé are serotonergic (adapted from Wang et al., 2001). Left panel: The firing rate of a single medullary raphé neuron increased in response to increased CO_2/decreased pH and decreased in response to decreased CO_2/increased pH. The increase in firing rate response was both reversible and repeatable. Right panel: Immunohistochemistry using an antibody for serotonin indicated the neuron (arrow head) was serotonergic.

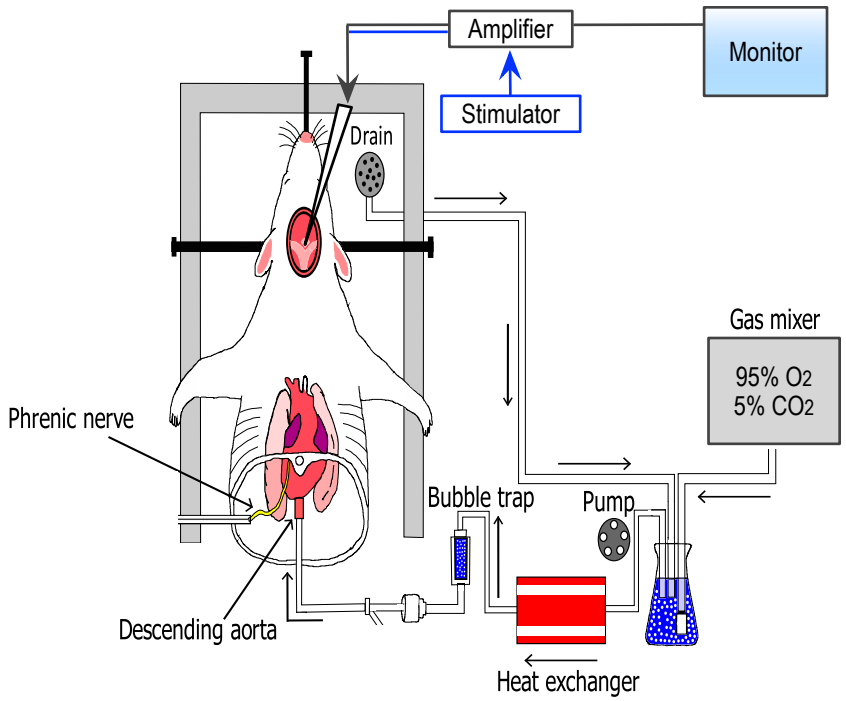

Figure 1.7. Diagram of the *in situ* perfused brainstem preparation. Shown is a bisected, decerebrated rat in prone position in the recording chamber. Perfusate solution was pumped through a heat exchanger and bubble trap, entered the preparation through the descending aorta, and was recirculated. Levels of O_2 and CO_2 were controlled by a gas mixer bubbling gas directly into the perfusate reservoir. A recording electrode was placed in the midline of the brainstem, and action potentials were amplified and monitored by computer output. The juxtacellular labeling method was employed using a stimulator to inject current through the recording electrode. A complete description of methods is given in the appendix. Credit for drawing to Bryan Mosher, Harris laboratory, University of Alaska Fairbanks.

Chapter 2

Isoflurane dose-dependently inhibits medullary raphé 5-HT neurons *in situ*[1]

2.1 Abstract

The ascending and descending serotonin (5-HT) networks are critically involved in the motor, somatosensory, and limbic systems and as such, serotonergic neurons are the subject of much research in a wide range of fields. The majority of *in vivo* electrophysiological studies of 5-HT neurons are conducted in animals maintained under anesthesia, and yet little is known about how anesthetics affect 5-HT neuron function. Here we used electrophysiology in the unanesthetized *in situ* perfused brainstem to determine how the volatile anesthetic isoflurane affects medullary raphé 5-HT neurons. We tested the hypothesis that medullary raphé 5-HT neurons are inhibited by isoflurane. We examined changes in 5-HT neuron baseline firing with 1, 1.5, and 2 % isoflurane, and we confirmed isoflurane concentrations by gas chromatography – mass spectrometry analysis. Results indicate that isoflurane inhibits 5-HT neurons, and this inhibition is dose-dependent. These findings will aid in interpretation of reports from *in vivo* studies of the 5-HT system conducted under anesthesia and may provide insight into how 5-HT neurons are involved in general anesthesia mechanisms.

[1] Johansen SL, Iceman KE, Iceman CR, Richerson GB, and Harris MB. 2014. Isoflurane dose-dependently inhibits medullary raphé 5-HT neurons *in situ*. In preparation for submission in the *British Journal of Anesthesiology*.

2.2 Introduction

The serotonergic system is an interconnected network of neurons that communicate via the amine neurotransmitter serotonin (5-hydroxytrptamine; 5-HT). Evolutionarily one of the oldest amine systems, these serotonergic cell bodies are located in the midbrain and caudal regions and project to virtually all areas of the central nervous system, allowing for their critical involvement in maintaining homeostatic processes across the motor, somatosensory, and limbic systems (Jacobs and Azmitia, 1992; Lovick, 1997; Hornung, 2003; Kinney et al., 2009). 5-HT neurons are divided into two populations based on their distinct anatomical location, connectivity, and function: the rostral group of the midbrain and pons and the caudal group of the medulla. The rostral 5-HT neurons (ascending serotonergic network), including the pontine raphé, dorsal raphé, and median raphé, project to the forebrain to mediate arousal, cognition, anxiety, and cerebral blood flow, and these neural circuits have been implicated in numerous neuropsychiatric and neurological disorders (Jacobs and Azmitia, 1992). The caudal 5-HT neurons (descending serotonergic network), including raphé obscurus, raphé pallidus, and raphé magnus in the midline and the parapyramidal region on the ventrolateral medullary surface (VLMS), project to the lower brainstem and spinal cord and mediate autonomic output, respiratory control, cardiovascular control, motor control, and nociception (Richerson, 2004). The widespread involvement of both ascending and descending serotonergic networks in diverse neural circuits leads to their hallmark characteristic as integrators, modulators, and critical regulators of essential homeostatic processes.

The majority of *in vivo* electrophysiological studies on the ascending and descending 5-HT networks in rodents are conducted in animals maintained under anesthesia, and yet little is known about how anesthetics affect 5-HT neuron activity. Maintaining animals under general anesthesia has research advantages that are not easily achieved in non-anesthetized preparations. From a technical standpoint, it is much easier to record from anesthetized than non-anesthetized animals. Some electrophysiological recordings of 5-HT neurons have been conducted in head-restrained but otherwise freely moving cats (Fornal et al., 1996; Veasey et al., 1997; Bjorvatn et al., 1998; Martin-Cora et al., 2005); however, chronically instrumenting these animals and achieving stable recordings while animals are moving remains a significant obstacle for successful data collection. Furthermore, anesthesia allows researchers to avoid changes in the behavioral state of the animal that could affect neuronal recordings. 5-HT neuronal firing spontaneously changes across the sleep-wake cycle (Bjorvatn et al., 1998), as do levels of neurotransmitters that regulate 5-HT neurons (Sakai and Crochet, 2000), and it would be difficult to distinguish behavioral-induced changes in 5-HT neuronal firing from experimental results. Additionally, drug treatments that affect movement, cognition, or anxiety level could change the behavioral state of the animal and effect neuronal recordings. Anesthesia allows for prolonged surgery with more stable electrophysiological recordings and avoids changes in behavioral arousal and cognitive state that could affect neuronal activity.

The research advantages of working under anesthesia are clear; however, before anesthesia can be used to facilitate 5-HT neuronal recordings in animals, it is necessary to define how these anesthetics affect 5-HT neuron function. Prior studies on anesthetics and 5-HT neurons have primarily focused on how 5-HT neurons contribute to the state of anesthesia. The ligand-gated 5-HT_3 receptor, which is highly sensitive to modulation by volatile anesthetics (Franks and Lieb, 1994; Campagna et al., 2003; Mashour et al., 2005), is postulated to contribute to producing general anesthesia (Jenkins et al., 1996) and may also contribute to postoperative side effects (Haigh et al., 1993). Previous reports documenting that inhalational anesthetics block the effect of the neurotransmitter serotonin on 5-HT_{2A} and/or 5-HT_{2C} receptors, receptor subtypes known to be involved in anti-nociception in the spinal cord (Bardin et al., 2000), suggest that 5-HT neurons contribute to analgesia and immobility during anesthesia (Minami et al., 1997; Zhang et al., 2003; Nagatani et al., 2011). Serotonin release in the frontal cortex decreases during isoflurane anesthesia, similar to slow-wave sleep, which suggests a role for 5-HT neurons in hypnosis during anesthesia (Mukaida et al., 2007). These prior studies indicate that anesthetic modulation of serotonergic transmission contributes to the behavioral effects of general anesthesia.

Although it has been clearly demonstrated that anesthetics modulate serotonergic transmission, most notably through postsynaptic action on 5-HT receptors, surprisingly few studies target the presynaptic effects of anesthetics on 5-HT neurons themselves. McCardle and Gartside (2012) studied the influence of a range of anesthetics on dorsal

raphé 5-HT neuron activity *in vitro*. However, this study did not investigate how 5-HT neuron firing is affected by the commonly used volatile anesthetic isoflurane, a halogenated ether compound that, due to its low blood solubility leading to faster elimination time compared to many other general anesthetics, is widely used in both clinical and scientific contexts (Campagna et al., 2003). Additionally, the effects of general anesthetics on caudal 5-HT neurons in the medullary raphé and VLMS have not been systematically investigated. Thus, the effect of anesthesia on 5-HT neuron function is not wholly defined.

Importantly, studying the effects of anesthetics on 5-HT neurons themselves contributes to the growing body of literature that suggests that presynaptic mechanisms are critical sites of anesthetic action. Until recently, the majority of research on anesthetic mechanisms has focused on postsynaptic sites of anesthetic action, notably on $GABA_A$ receptors, but presynaptic effects on transmitter release are also identified (Griffiths and Norman, 1993; Pocock and Richards, 1993). Current evidence suggests that volatile anesthetics exert presynaptic effects on voltage-gated Na^+ channels (Herold and Hemmings, 2012), two-pore-domain background K^+ channels (Bayliss and Barrett 2008), and voltage-gated Ca^{2+} channels (Orestes and Todorovic, 2010), all of which could affect 5-HT neuron activity under anesthesia. The presynaptic effect of anesthetics, specifically on 5-HT neuron function, remains a pertinent question.

We have previously shown that the anesthetic isoflurane disrupts the pH/CO_2 chemosensitivity of a subset of medullary raphé 5-HT neurons that are proposed to function as central chemoreceptors in respiratory control (Johansen et al., 2012; Massey et al., 2013). In the present study, we characterized the effect of isoflurane on baseline firing of medullary raphé 5-HT neurons using the arterially perfused *in situ* brainstem preparation. We tested the hypothesis that isoflurane inhibits baseline firing of medullary raphé 5-HT neurons *in situ*. We demonstrated the influence of isoflurane on 5-HT neuron firing rate and evaluated the dose-dependence of isoflurane-induced 5-HT neuron inhibition within a clinically relevant range (1, 1.5, and 2 %), in which 1 % isoflurane approximates 1.15 MAC (minimum alveolar concentration that prevents response to noxious stimulation in 50 % of subjects; Eger, 1981). We confirmed the dose concentration by gas chromatography – mass spectrometry analysis. Our present aim is to further define the effect of isoflurane on 5-HT neuron activity. Our findings will aid in interpretation of results from *in vivo* studies that use anesthesia, contribute to the growing body of literature describing presynaptic mechanisms of anesthetic action, and may provide insight into how 5-HT neurons are involved in mechanisms of general anesthesia.

2.3 Methods

Experimental animals and preparations: Animal care and experimental procedures followed guidelines set by the National Institutes of Health Office of Laboratory Animal Welfare and the United States Department of Agriculture Animal Welfare Act. All experiments were done in accordance with the guidelines of the "Guide for the Care and

Use of Laboratory Animals" of the National Institutes of Health and were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee. Juvenile male rats (50 – 150 g; Sprague-Dawley strain; Simonson Laboratories) were used to generate the perfused *in situ* brainstem preparation as previously described (Paton, 1996; Corcoran et al., 2013; Iceman et al., 2013). Briefly, animals were administered an intraperitoneal heparin sodium injection (0.5 mL, 1000 USP; Sagent Pharmaceuticals) to prevent formation of blood clots during surgery. Deep anesthesia was induced using isoflurane and assessed by cessation of spontaneous breathing and absence of a withdrawal response to firm toe pinch. Anesthesia was discontinued as preparations were bisected sub-diaphragmatically and decerebrated rostral to the superior colliculi, and subsequent procedures were conducted in the absence of anesthesia. Preparations were immersed in chilled artificial cerebral spinal fluid (aCSF) for the remainder of the dissection, which was conducted to isolate the descending aorta.

Each preparation was placed in the recording chamber in a prone position. The descending aorta was cannulated retrogradely with a double-lumen catheter, and preparations were perfused with a solution at a temperature of 31 °C. The perfusate solution contained the following (in mM): 1.0 $MgSO_4$, 125 NaH_2PO_4, 4.0 KCl, 24 $NaHCO_3$, 115 NaCl, 10 D-Glucose, 2.0 $CaCl_2$, and 0.18 Ficoll. Baseline conditions, when the perfusate was equilibrated with 95 % O_2 – 5 % CO_2 (PCO_2 33 mmHg; pH=7.4), approximated normocapnic plasma *in vivo*. Levels of O_2 and CO_2 in the perfusate were controlled by equilibrating a perfusate reservoir with gas mixtures produced by a

precision gas mixer (CWE GSM-3) and verified by a CO_2 analyzer (Applied Electrochemistry CD-3A). Following cannulation, perfusion pressure was gradually increased to 40-60 mmHg by regulating the speed of a peristaltic perfusion pump. The first 50 mL of perfusate that passed through the preparation was discarded to eliminate blood, and the remaining 450 mL of perfusate was recirculated. The neuro-muscular blocker gallamine triethidodide (60 mg/L, Sigma-Aldrich) was added to the perfusate to eliminate movement. The occipital bone, surrounding musculature, and cerebellum were removed to expose the dorsal surface of the brainstem.

Extracellular recording and experimental treatments: Extracellular recordings of medullary raphé (r. magnus, r. obscurus, r. pallidus) neurons were made using pulled-glass capillary electrodes (15–20 MΩ) filled with biotinamide hydrobromide (5 % in 0.5 M sodium acetate, Life Technologies). The electrode was positioned along the brainstem midline (≤ 0.1 mm lateral) 0 to 3 mm rostral of obex and advanced through the brain tissue in 2 μm increments using a fine stepping motor (Burleigh Instruments Inchworm). Extracellular recordings were made with an intracellular amplifier (Axon Instruments Multiclamp 700B) in current clamp mode, with a high pass filter at 300 Hz and low pass filter at 1 kHz bessel with an high impedance headstage (Axon CV7B, Molecular Devices). Action potentials were digitized using Spike 2 (Cambridge Electronic Design Power 1401) and LabChart (AD Instruments) software and stored as computer data files.

Neuronal recordings were initiated under baseline conditions (95 % O_2 – 5 % CO_2; PCO_2 33 mmHg; pH=7.4). As stated, these conditions approximate normocapnic plasma *in vivo*; however, due to the lack of hemoglobin, the solution hyperoxia (PO_2 ~ 600 mmHg) was necessary to maintain O_2 content sufficient to meet tissue metabolic demands, and this unavoidable hyperoxia was constant under all conditions. The normocapnic condition was followed by a hypercapnic challenge (91 % O_2 – 9 % CO_2; PCO_2 60 mmHg; pH 7.2; 5 min) to approximate conditions similar to those in plasma during a 4 % increase in inspired CO_2. The preparation was then returned to normocapnic conditions for an additional 5 min. The 5-min exposure was demonstrated to be sufficient for brain tissue equilibration with the 91 % O_2 – 9 % CO_2 gas levels in the perfusate (Wilson et al., 2001).

Spontaneously active neurons were identified electrophysiologically and classified according to changes in firing frequency elicited by elevation of arterial CO_2 concentrations during hypercapnic challenges (increase, decrease, and no change in baseline firing frequency). Both chemosensitive and insensitive neurons were administered isoflurane bubbled into the perfusate for 10 min (1, 1.5, or 2 % isoflurane in 95 % O_2 – 5 % CO_2; percent isoflurane refers to volume percent). For chemosensitive neurons, the 5-min hypercapnic challenge was repeated in the presence of isoflurane (1, 1.5, or 2 % isoflurane; 91 % O_2 – 9 % CO_2). For insensitive neurons, hypercapnic challenges were not repeated. Upon return to baseline conditions (0 % isoflurane; 95 % O_2 – 5 % CO_2), the neuron was allowed to recover toward its baseline firing frequency.

Recovery times ranged from 10-30 min, depending on the individual neuronal characteristics and assays performed.

Juxtacellular labeling: Subsets of recorded neurons were individually filled with biotinamide using the juxtacellular labeling method (Pinault, 1996; Winkler et al., 2006). Extracellular recordings were conducted in current clamp mode (Axon Multiclamp 700B) to allow current to be injected through the electrode while action potentials were monitored. Positive current pulses were applied through the electrode (400-ms duration) and gradually increased from 0-15 mV (0.5 mV steps; Grass Stimulator S44B) until the cell was entrained to fire simultaneously with the applied stimulus. Injected current, ejection of biotinamide, and cellular firing entrainment caused uptake of the biotinamide marker by the recorded cell. Entrainment was maintained for at least 30 s. After termination of entrainment, the biotinamide was allowed to disperse for 30 min. Rats were then perfused through the descending aorta with fixative [4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer saline (PBS); pH 7.4; Sigma-Aldrich]. Brainstems were then removed and stored in fixative prior to sectioning. Coronal sections (60 μm) were cut through the medulla (Vibratome 1000Plus), and sections were processed free-floating.

Immunohistochemistry: Coronal tissue sections were blocked and permeabilized in blocking buffer [Blocking buffer: 5 % normal goat serum (NGS in 0.1 % sodium axide; Caltag Spec. No. L10000, Lot: 384935A) and 0.3 % Triton X-100 in 0.1 M PBS] for 1 hr at 22 °C. Sections were incubated with a streptavidin-Alexa 546 conjugate (Life

Technologies #S-11225; 1:500 dilution in PBS with 5 % NGS) for 2 hr at 22 °C to reveal biotinamide introduced into single neurons by juxtacellular labeling. To identify serotonergic cells, we used an antibody for the 5-HT-synthesizing enzyme tryptophan hydroxylase (TpOH). Sections were incubated overnight at 4 °C with mouse monoclonal anti-TpOH (Sigma-Aldrich #T0678; 1:1000 dilution in blocking buffer). Following incubation for 1 hr at 22 °C, sections were incubated for 2 hr at 22 °C in a secondary goat anti-mouse Alexa 488 antibody (Life Technologies #A-11029; 1:500 dilution in 0.1 M PBS with 5 % NGS) to label TpOH-immunoreactive cells. Sections were air-dried for 30 min, mounted with Vectashield (Vector Labs), and coverslipped. Low-magnification (10x) images were used to determine the location of biotinamide-labeled cells in relation to anatomical landmarks. Local biotinamide and 5-HT fluorescence identified colocalization of serotonergic markers in biotinamide-labeled neurons. Fluorophores were individually excited and emission spectra were collected separately to minimize interference using a Zeiss LSM510 confocal microscope: biotinamide-labeled neuron, Alexa 546, 543 nm laser, filter BP 560-615; anti-TpOH, Alexa 488, 488 nm laser, filter BP505-530. Images were constructed as 40x projections of z-stacks.

Gas chromatography – mass spectrometry: All gas chromatography – mass spectrometry (GC – MS) analyses were performed on an Agilent Technologies 7890A GC System and 5975C mass selective detector with Triple Axis Detector. Helium carrier gas was used at a constant flow rate of 20 mL min^{-1}. A sample of 250 μL was injected into a DB-1 ms capillary column (45 m x 0.25 mm, film thickness 0.25 μm) at a split ratio of 50:1. The

system oven temperature was initially maintained at 30 °C for 6 min, and then increased at a rate of 10 °C min^{-1} from 30 to 120 °C. MS acquisition was performed in full scan mode in the 40 to 200 amu mass ranges. Mass conditions were as follows: ionization voltage, 69.9 eV; ion source temperature, 230 °C; detector voltage, 1.6 kV.

A saturated solution of isoflurane was prepared by adding aliquots of isoflurane to perfusate solution and stirring in an airtight container for 3 hr (15 mM; Scheller et al., 1997). Different concentrations were prepared by diluting the saturated solution in perfusate (1:2, 1:4, 1:8, 1:16, 1:32, and 1:64) and 250 μL of headspace gas from each dilution was injected directly onto the GC – MS. The mean peak area for each dilution, corresponding to the number of isoflurane molecules in solution, was used to generate a standard curve for isoflurane. The standard curve was fit to a linear regression line (SigmaPlot).

Samples of perfusate were taken from the aortic cannula, to best approximate the sample received by experimental preparations, at each minute during a 10-min 1 % isoflurane exposure. Additional samples were taken at 11 and 15 min to confirm that isoflurane concentrations were stable throughout the period of recording. GC – MS analysis of these samples yielded a value for mean peak area, corresponding to the number of isoflurane molecules in solution. The concentration of isoflurane in solution at each minute of the drug exposure was calculated from the standard curve equation. These calculated concentrations were used to confirm that at 10 min, the time point when all neuronal

responses to isoflurane were recorded, the concentration of isoflurane in the perfusate was equivalent to 1 volume percent.

Data analysis: We discriminated extracellularly recorded individual unit activity using computer spike sorting software (Spike Histogram, AD Instruments). Stable 1-2 min periods of individual unit firing were analyzed before and during hypercapnic challenge to provide a mean value for unit firing frequency (spikes/s) and interspike interval (ms). Subset analysis was used to classify neurons as chemo-stimulated or chemo-inhibited where relative frequencies changed by greater than 20 %, and chemo-insensitive where relative frequencies changed by less than 20 % (Wang et al., 2001). We have previously reported the influence of isoflurane on hypercapnic responses in chemo-stimulated 5-HT neurons (Johansen et al., 2012; Massey et al., 2013). The present data set includes only chemo-insensitive neurons.

Cells were initially screened for being serotonergic by a real-time assessment of firing frequency and regularity. Neurons with firing frequencies between approximately 0.5 and 3 Hz that fired regularly, without sustained pauses or bursts in firing, were regarded as putative serotonergic neurons during experimental recording. Putative serotonergic cells were confirmed as such with the following algorithm, in which the mean interspike interval ($\overline{X}$, in ms) and standard deviation of the intervals (sd, in ms) are considered independent variables: $Y(\overline{X},sd) = 146 - \overline{X} + 0.98sd$ (Mason, 1997). If the value of this function is less than zero [$Y(\overline{X},sd) < 0$], then the cell was confirmed serotonergic, and if

the value of the function is greater than zero [Y($\overline{X}$,sd) > 0], then the cell was considered non-serotonergic. All putative 5-HT neurons that were identified by real-time assessment of firing pattern and regularity for recording were confirmed serotonergic cells by the algorithm.

All putative 5-HT neurons (n=38) were administered a single dose of isoflurane [1 % (n=18); 1.5 % (n=10); or 2 % (n=10)]. For each dose, the mean firing frequency at baseline (0 % isoflurane) and mean firing frequency in the last minute of isoflurane exposure (1, 1.5, or 2 % isoflurane) were compared using a paired *t*-test. Baseline firing frequencies for each of the three groups (1, 1.5, and 2 % isoflurane) were compared by one-way ANOVA to confirm that mean firing frequencies under baseline conditions were not significantly different from each other. To assess the existence of a dose response, percent change from baseline during isoflurane exposure was compared between isoflurane treatment groups using a one-way ANOVA. Post-hoc tests used Holm-Sidak pairwise comparison (SigmaPlot). Throughout the text, values are expressed as means ± standard error of the mean. The criteria for statistical significance was $P < 0.05$, and instances of $P < 0.01$ and $P < 0.001$ are also noted.

2.4 Results

Isoflurane inhibits firing of medullary raphé 5-HT neurons. Single unit extracellular recordings performed on medullary raphé putative 5-HT neurons before, during, and after isoflurane treatment indicated that isoflurane inhibited the action potential discharge of

these neurons. Putative 5-HT neurons were characterized by firing frequency and regularity (Mason, 1997). Subsets of these neurons were filled with biotinamide and confirmed serotonergic neurons by colocalization of markers of serotonin synthesis [presence of tryptophan hydroxylase (TpOH)] in the biotinamide-labeled neuron. A representative cell is shown in Figure 2.1. Putative 5-HT neurons exhibited a baseline firing frequency within the range 0.1 to 2.01 Hz and a firing pattern consistent with serotonergic neuron characteristics, including a large spike width, long after-hyperpolarization, and regular firing without any sustained pauses or bursts in firing. Putative 5-HT neurons either decreased or ceased firing during isoflurane treatment and returned to baseline upon washout. Following recovery, a subset of these neurons was entrained to fire simultaneously with an applied stimulus. Application of stimulus via the recording electrode caused extrusion of biotinamide from the electrode's capillary barrel. Cellular firing entrainment resulted in uptake of this biotinamide label. Subsequent immunohistochemistry confirmed the presence of TpOH, an enzyme unique to 5-HT synthesis, and thus, a marker of serotonergic neurons, in biotinamide-labeled neurons, which confirmed the neurons were serotonergic. Thus, a number of recorded neurons were confirmed to be 5-HT neurons, and the remainder were confirmed as 5-HT neurons by the algorithm that distinguishes between serotonergic and non-serotonergic cells using firing pattern characteristics (Mason, 1997).

Isoflurane inhibits firing of raphé 5-HT neurons at different concentrations. All putative 5-HT neurons (n=38) were administered a single dose of isoflurane at

concentrations approximating 1.15 MAC (minimum alveolar concentration for isoflurane): 1 % (n=18); 1.5 % (n=10); or 2 % (n=10). 36 of the 38 recorded cells were inhibited or silenced by isoflurane. For 2 of the 38 recorded cells, baseline firing either did not significantly change (7.89 % increase) or significantly increased (48.0 % increase) in response to 1 % isoflurane (data not shown). These neurons were positively identified as 5-HT by the algorithm (Mason, 1997) and were included in all data analysis. The mean firing frequency in the last minute of isoflurane exposure for each isoflurane dose (1 % isoflurane: 0.349 ± 0.11 Hz; 1.5 % isoflurane: 0.113 ± 0.04 Hz; or 2 % isoflurane: 0.021 ± 0.01 Hz) was significantly different from the mean firing frequency under baseline conditions prior to isoflurane exposure (0 % isoflurane: 0.965 ± 0.07 Hz), as determined by paired *t*-test ($P < 0.001$), which indicated that isoflurane inhibits medullary raphé 5-HT neurons at a range of concentrations approximating 1.15 MAC (Fig. 2.2). A one-way ANOVA confirmed that mean firing frequencies at baseline for each isoflurane treatment group were not significantly different ($P = 0.971$).

Isoflurane-induced inhibition of raphé 5-HT neurons is dose-dependent. The mean percent decrease from baseline during isoflurane exposure was calculated for each isoflurane treatment group (1 % isoflurane: -68.96 ± 9.57 %; 1.5 % isoflurane: -89.78 ± 3.52 %; 2 % isoflurane: -97.76 ± 1.24 %). A comparison of these mean percent changes using one-way ANOVA indicated a significant difference between means for neurons administered 1 % and 2 % isoflurane (means differed by 28.79 %; $P < 0.05$), which confirmed that isoflurane-induced inhibition of putative raphé 5-HT neurons was dose-

dependent (Fig. 2.3). The percent changes induced by 1 and 1.5 % isoflurane were not significantly different (p=0.147) and neither were the changes induced by 2 and 1.5 % isoflurane (p=0.541).

Presence of isoflurane in the perfusate confirmed by gas chromatography – mass spectrometry analysis. Aliquots of the perfusate sampled during administration of isoflurane were analyzed by gas chromatography – mass spectrometry (GC – MS) to confirm that the experimental preparation received the intended concentration of isoflurane via the perfusate. A chromatogram of a sample of 1 % isoflurane in extracellular solution indicates two distinct peaks (Fig. 2.4a). The peaks at retention times 2.64 and 3.20 min were identified as CO_2 and isoflurane, respectively, by mass spectral analysis. As shown in Figure 2.4b, the mass spectra for isoflurane ($C_3H_2ClF_5O$; molecular weight = 184.49 g/mol) showed characteristic molecular and fragment ions: m/z 149 ($C_3H_2F_5O$ – Cl) and 116.9 (C_2HClF_3 – $OCHF_2$). The mass spectra for CO_2 showed the characteristic peak at m/z 44 (Fig. 2.4c).

Standard curve for isoflurane based on GC – MS analysis of perfusate. A standard curve for isoflurane was generated from a saturated solution of isoflurane (15 mM; Scheller et al., 1997), and dilutions of the saturated solution (1:2, 1:4, 1:8, 1:16, 1:32, and 1:64) were analyzed by GC – MS. The mean peak area for each dilution, corresponding to the number of isoflurane molecules in solution, was used to generate the standard curve for isoflurane (Fig. 2.5), which was fit to a linear regression line described by the

following equation: $y = 6.2*10^6 x + 6.0*10^5$ ($R^2 = 0.994$).

Isoflurane concentration in perfusate is equivalent to 1 volume percent. Isoflurane is poorly soluble in water, but readily soluble in plastic and rubber tubing (Eger, 1981); thus, it was imperative that we confirm the concentration of isoflurane received by the preparation through the perfusate. GC – MS analysis of perfusate samples taken at each minute during a 10-min 1 % isoflurane exposure yielded a value for mean peak area, corresponding to the number of isoflurane molecules in solution. The concentration of isoflurane in solution at each minute of the drug exposure was calculated from the standard curve equation: $y = 6.2*10^6 x + 6.0*10^5$ ($R^2 = 0.994$). At 10 min, the time point when all neuronal responses to isoflurane were recorded, the mean equilibrium isoflurane concentration was 0.38 mM, which was equivalent to 1 volume percent (0.38 mM = mean concentration from 6 to 10 min; 0.37 mM = 1 volume percent; Eilers et al., 1999). The 6 to 10 min range was chosen to calculate the mean equilibrium concentration because the concentration steadily increased from 3 to 6 min and remained relatively constant from 6 to 10 min. Additional samples taken at 11 and 15 min confirmed the stability of isoflurane concentrations throughout the period of recording.

2.5 Discussion

Here we present the first evidence that isoflurane inhibits the baseline firing of caudal raphé 5-HT neurons in the descending 5-HT system. Isoflurane routinely inhibited the baseline firing of 5-HT neurons, or caused firing to cease completely. These results were

consistent for all tested concentrations of isoflurane (1, 1.5, and 2 %), and the degree of inhibition increased as the concentration of isoflurane in extracellular solution increased. We found a significant difference between neuronal responses to 1 and 2 % isoflurane, which indicated dose responsiveness of 5-HT neurons to isoflurane within a clinically relevant range. 1 % isoflurane approximated 1.15 MAC, the minimum alveolar concentration of isoflurane necessary for surgical anesthesia (Eger, 1981). This concentration was confirmed by gas chromatography – mass spectrometry analysis. This analysis allowed us to confirm that our results from *in situ* experiments are relevant to evaluating results from *in vivo* experiments on anesthetized animals, which typically are anesthetized within the range of 1 to 2 % inhaled anesthetic. Furthermore, confirming that the concentration of isoflurane received by the preparation was equivalent to 1.15 MAC allowed us to ensure that our results documenting the influence of isoflurane on 5-HT neurons are clinically relevant.

For 2 of the 38 cells recorded in this study, baseline firing either did not significantly change or significantly increased in response to 1 % isoflurane. These cells were positively identified as 5-HT neurons by the Mason algorithm (Mason, 1997) and were included in all data analysis. The cell that did not respond to 1 % isoflurane may not have expressed anesthetic-sensitive channels or the effect of the anesthetic may have been masked by other network inputs. The cell that increased firing in response to isoflurane may have experienced disinhibition as part of a network that was inhibited by isoflurane. Regardless of the mechanism underlying these two unique responses, even when these

cells were included in the composite data set, the collective results indicate that isoflurane inhibits 5-HT neurons.

The mechanism by which isoflurane inhibits 5-HT neurons remains a pertinent research question, but evidence does support a specific binding site for isoflurane on two-pore domain potassium channels (Bayliss and Barrett, 2008). These channels mediate instantaneous open-rectifier background K^+ currents, and volatile anesthetics have been shown to potentiate this background current (Sirois et al., 1998; Patel et al., 1999; Sirois et al., 2000; Talley et al., 2000). Two subtypes of TASK channels, TASK-1 and TASK-3, are highly expressed in the raphé nuclei compared to other brain regions (Talley et al., 2001). Specific to raphé serotonergic cells, the majority of 5-HT neurons (70-90 %) in both dorsal and caudal raphé cell populations have been shown to express TASK-1 and TASK-3 channels (Washburn et al., 2002). Based on these prior studies, we postulate that potentiation of the TASK channel-mediated background K^+ current by isoflurane may have hyperpolarized our target population of raphé 5-HT neurons and caused their baseline firing frequencies to decrease or cease firing during isoflurane treatment.

Presynaptic binding sites for volatile anesthetics on voltage-gated Na^+ channels have also been reported (Hemmings, 2009; Herold and Hemmings, 2012) and voltage-gated Ca^{2+} channels (Orestes and Todorovic, 2010). Blockade of Na^+ channels by anesthetics has been shown to inhibit action potential conductance (Ouyang and Hemmings, 2005; Wu et al., 2004), and anesthetic-induced inhibition of Ca^{2+} currents has been shown to depress

membrane excitability and neurotransmitter release (Study, 1994; Nikonorov et al., 1998; Kamatchi et al., 1999). The influence of isoflurane on these channels that mediate action potential conductance and neuronal excitability could also contribute to the inhibition of action potential discharge in 5-HT neurons that we observed in response to isoflurane.

Our data demonstrating that isoflurane inhibits action potential generation in 5-HT neurons suggest that anesthetics act on presynaptic sites, a hypothesis that, until recently, has been overshadowed by research on postsynaptic anesthetic effects. Many studies on the influence of anesthetics on 5-HT neurons have focused on the molecular action of anesthetics on postsynaptic 5-HT receptors (Minami et al., 1997; Bardin et al., 2000; Mukaida et al., 2007; Nagatani et al., 2011). Importantly, our recordings of 5-HT neuron action potentials allow for observation of presynaptic effects during anesthesia, and our results demonstrating that isoflurane inhibits 5-HT neuron firing contribute to the growing body of literature demonstrating that presynaptic sites are also critical to anesthetic action (Griffiths and Norman, 1993; Pocock and Richards, 1993). Although we did not directly measure neurotransmitter release from the synaptic terminal, the inhibition of 5-HT neuron firing observed during isoflurane treatment is assumed to result in decreased action potential dependent release of serotonin. These observations are consistent with the generally accepted hypothesis that anesthetic-induced neuronal depression results from enhanced inhibitory transmission and decreased excitatory transmission (Franks and Lieb, 1994; Campagna et al., 2003; Torri, 2010). Further investigations into presynaptic influences of isoflurane on 5-HT neurons may have

important implications in our understanding of the neurological mechanisms governing 5-HT neuron involvement in the state of anesthesia.

The effect of anesthetics on dorsal raphé 5-HT neuron activity in the ascending 5-HT system has been previously characterized (McCardle and Gartside, 2012), but anesthetic influences on caudal raphé 5-HT neuron activity in the descending 5-HT system had not been investigated prior to this study. The caudal raphé population of 5-HT neurons that we targeted mediate autonomic output, respiratory control, cardiovascular control, motor control, and nociception (Richerson, 2004). Inhibition of this serotonergic system by isoflurane may contribute to the immobilization, sedation, insensitivity to pain, and cardiorespiratory depression characteristic of isoflurane anesthesia. In fact, several reports suggest that the serotonergic system contributes to general anesthetic effects, including analgesia, immobility, and hypnosis. 5-HT_{2A} and 5-HT_{2C} receptor blockade modulated sevoflurane-induced immobility in response to noxious stimulation in rats *in vivo*, suggesting a role for 5-HT neurons in the immobility effects of anesthesia (Nagatani et al., 2011). Volatile anesthetics blocked the response of 5-HT_{2A} receptors to the excitatory neurotransmitter serotonin *in vitro* (Minami et al., 1997), and 5-HT_{2A} receptors were shown to mediate 5-HT-induced analgesic effects in rats *in vivo* (Bardin et al., 2000), which may indicate 5-HT neuron involvement in pain tolerance during anesthesia. *In vivo* microdialysis measuring extracellular 5-HT indicated that 5-HT release decreased in the rat frontal cortex during isoflurane anesthesia to a similar degree as during slow-wave sleep, suggesting that 5-HT neurons may contribute to hypnosis (Mukaida et al.,

2007). Collectively, these data indicate that decreased serotonergic transmission contributes to various states of anesthesia. Our data indicating that isoflurane inhibits 5-HT neuron firing and, therefore, action potential-dependent release of serotonin is consistent with this mechanism, in which decreased serotonergic transmission, whether by postsynaptic receptor blockade or inhibited presynaptic neuronal firing, gives rise to the state of anesthesia.

Defining the molecular mechanisms that give rise to the state of anesthesia holds broad interest and impact clinically and, since anesthetics are also abundantly used in scientific practice, elucidating how anesthetics affect neuronal activity holds great practical significance in interpretation of scientific findings and experimental design. The majority of *in vivo* electrophysiology studies on 5-HT neurons are conducted in animals maintained under anesthesia. Given that we observe marked inhibition of 5-HT neurons in response to isoflurane, it is plausible that *in vivo* experiments on 5-HT neuron activity using anesthetized rodents unknowingly target only those 5-HT neurons that are marginally sensitive or insensitive to isoflurane. These populations may express low levels of TASK channels, or isoflurane-insensitivity may be conferred by a different unknown mechanism. The effect of isoflurane on 5-HT neurons observed in this study may apply to other inhaled anesthetics that are currently used in clinical and research practice, including halothane, enflurane, sevoflurane, and desflurane (Campagna et al., 2003). It would be informative for both clinical and scientific purposes to systematically investigate the effects of different types of anesthetics on 5-HT neuron activity. Our

results demonstrating that isoflurane causes hyperpolarization of 5-HT neurons *in situ* are critical to consider when interpreting results from *in vivo* experiments on anesthetized rats. Although the research advantages of using anesthesia for *in vivo* electrophysiology are clear, the present data should be taken into account when designing future experiments to study 5-HT neuron activity *in vivo*.

Our study was limited by the difficulty of cellular entrainment for subsequent identification by neurotransmitter phenotype. Although it would certainly be ideal to conclusively identify all recorded cells, the juxtacellular labeling technique is by nature a relatively low-yield process. However, we targeted areas in the medullary raphé that are known to contain high concentrations of serotonergic cell bodies, and we selected cells that were likely to be serotonergic based on characteristic firing pattern and regularity. We identified serotonergic cells for recording by their characteristic slow rhythm without pauses or bursts, large spike width, and long after-hyperpolarization. All recorded cells are analyzed by an algorithm that distinguishes between serotonergic and non-serotonergic cells (Mason, 1997). We have previously demonstrated the utility of using the juxtacellular labeling method in concert with this algorithm for 5-HT neuron identification (Iceman et al., 2013). In this study, all recorded 5-HT neurons were positively identified as serotonergic neurons by the algorithm.

Future studies should target the question of how anesthetics affect 5-HT neurons presynaptically. We postulate that inhibition of the action potential results in decreased 5-

HT release, but this has not been confirmed by direct measurement of 5-HT release from synaptic terminals. Although we speculate that isoflurane potentiates the background K^+ current through TASK channels in 5-HT neurons, the mechanism by which isoflurane hyperpolarizes 5-HT neurons is not known. The extent to which inhibition of action potential discharge in 5-HT neurons by isoflurane contributes to the state of anesthesia is also unclear. Further investigation into the molecular action of isoflurane on 5-HT neurons and how this action relates to the behavioral effects of anesthesia is required.

In summary, we demonstrate that isoflurane inhibits baseline firing of 5-HT neurons in a concentration-dependent manner. Our work contributes to the growing body of literature that investigates presynaptic sites as critical components of mechanisms of anesthetic action. Documenting how anesthetics affect serotonergic neurons presynaptically is important for interpretation of scientific conclusions from experiments using anesthesia, and defining how this molecular action contributes to the state of anesthesia holds broad clinical relevance.

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2.7 Figures

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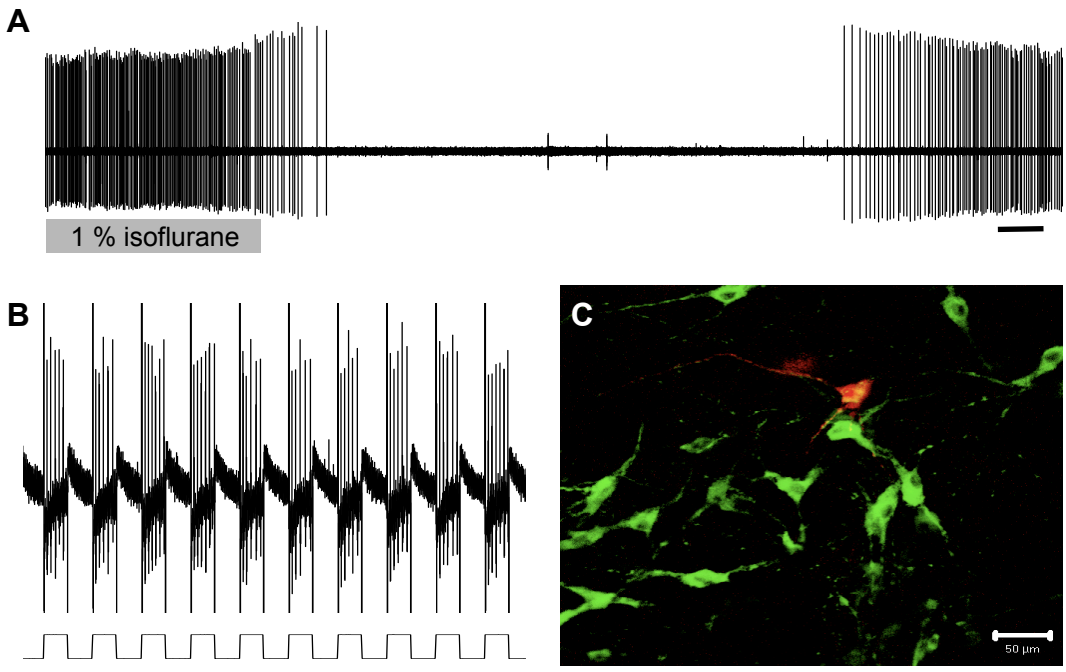

Figure 2.1. Isoflurane inhibits firing of medullary raphé 5-HT neurons. **a)** Representative extracellular recording of a 5-HT neuron that ceased firing during isoflurane treatment. The neuron returned to baseline upon washout. Under baseline conditions, firing frequency (1.12 Hz) and pattern was characteristic of serotonergic cells (Mason, 1997). Scale bar = 30 s. **b)** The same neuron was entrained to fire simultaneously with an applied stimulus, which extruded biotinamide and labeled the recorded neuron. **c)** Fluorescent markers of serotonin synthesis (tryptophan hydroxylase; green) and biotinamide (red) colocalize (yellow) to indicate the recorded neuron was a serotonergic neuron. Scale bar = 50 µm.

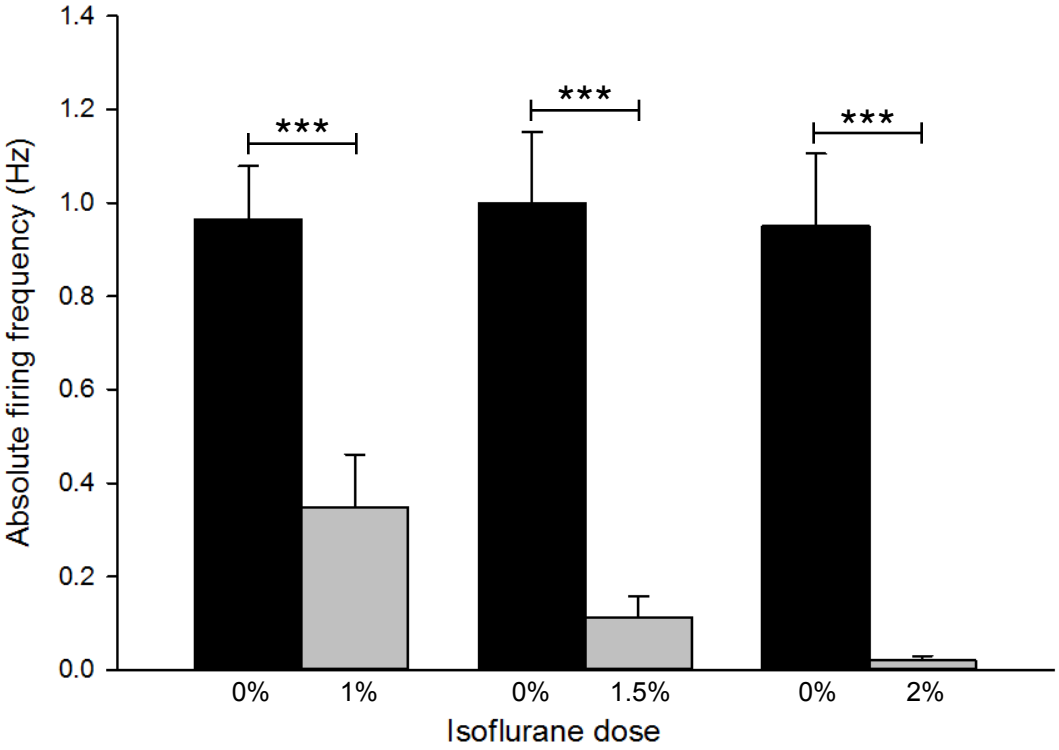

Figure 2.2. Isoflurane inhibits firing of raphé 5-HT neurons at various concentrations. All putative 5-HT neurons (n=38) were administered a single dose of isoflurane [1 % (n=18); 1.5 % (n=10); and 2 % (n=10)]. For each dose, comparison of mean firing frequency at baseline (0 % isoflurane; 0.965 ± 0.07 Hz) to mean firing frequency in the last minute of isoflurane exposure (1 % isoflurane: 0.349 ± 0.11 Hz; 1.5 % isoflurane: 0.113 ± 0.04 Hz; or 2 % isoflurane: 0.021 ± 0.01 Hz) was determined by paired *t*-test (***$P < 0.001$). Mean firing frequencies at baseline were compared by one-way ANOVA to confirm that baseline firing frequencies for the isoflurane treatment groups were not significantly different ($P = 0.971$).

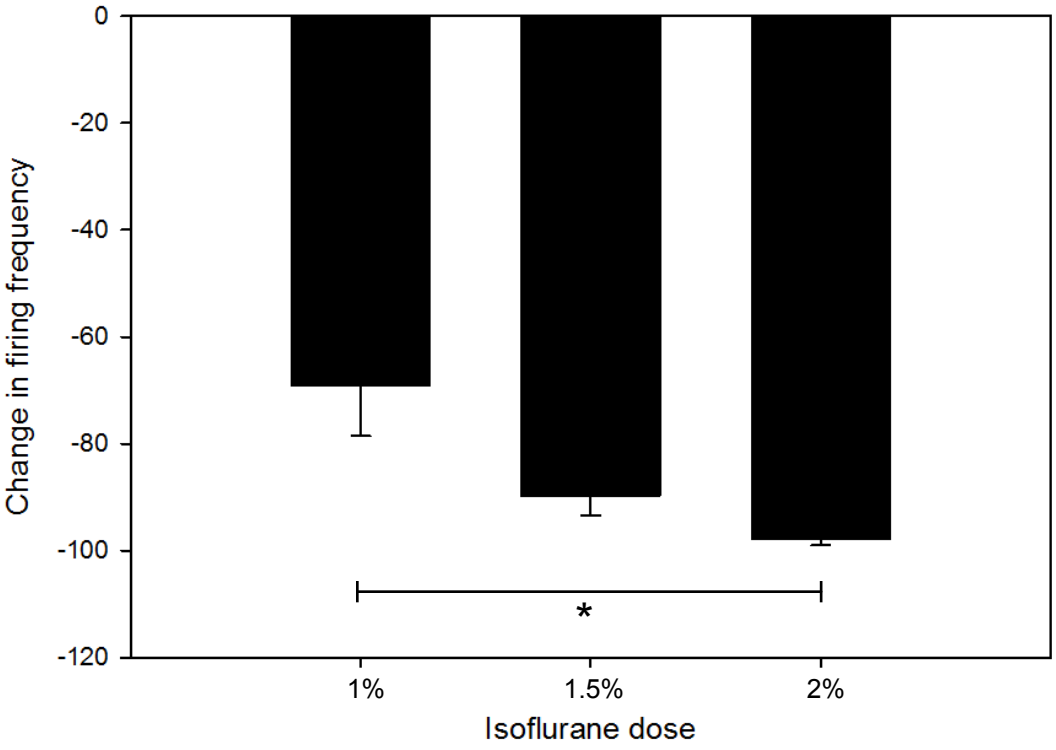

Figure 2.3. Isoflurane-induced inhibition of raphé 5-HT neurons is dose dependent. Values presented are mean percent decrease from baseline ± standard error for each isoflurane dose. Isoflurane doses of 1, 1.5, and 2 % inhibited raphé 5-HT neuron firing by 68.96 ± 9.57 (n=18), 89.78 ± 3.52 (n=10), and 97.76 ± 1.24 % (n=10), respectively. Between groups comparison of percent change from baseline during isoflurane exposure, using one-way ANOVA, indicated a significant difference between means for 1 and 2 % isoflurane (mean difference = 28.79 %; $*P < 0.05$). This analysis confirms that isoflurane-induced inhibition is dose-dependent. Between groups comparison of percent change from baseline was not significant comparing 1 and 1.5 % isoflurane ($P = 0.147$) or comparing 1.5 and 2 % isoflurane ($P = 0.541$).

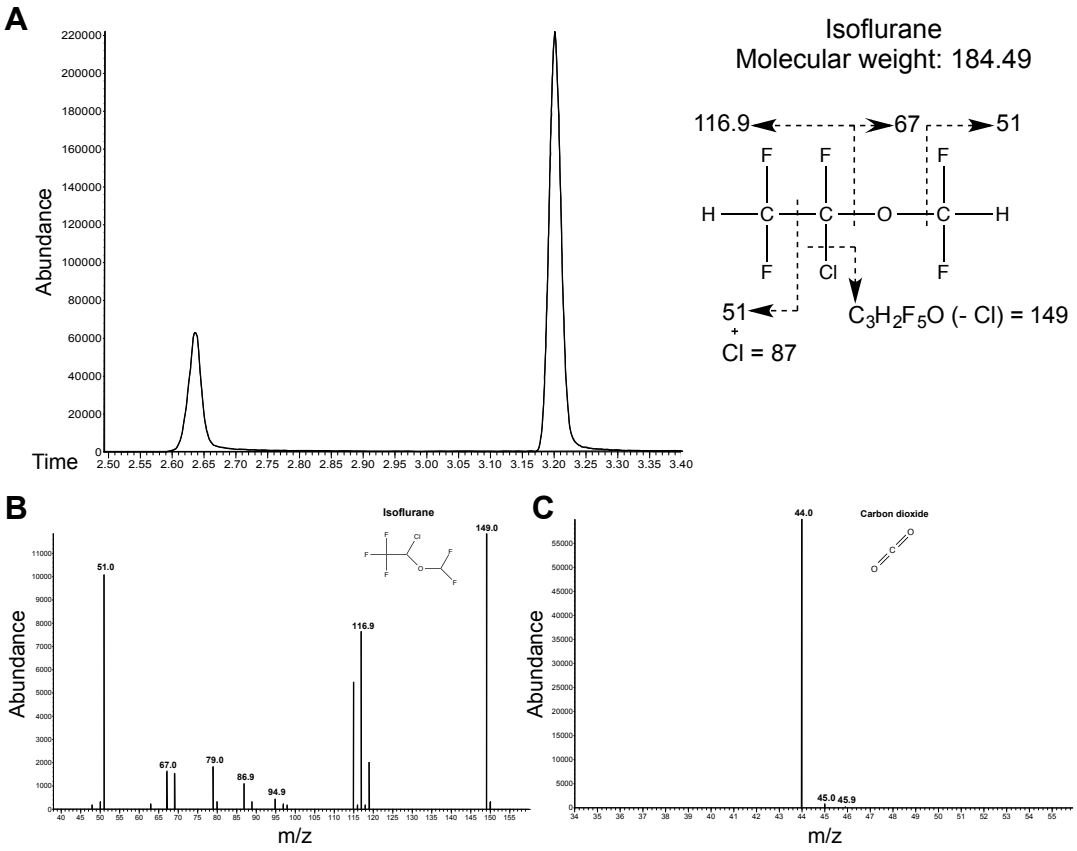

Figure 2.4. Presence of isoflurane in the perfusate is confirmed by gas chromatography – mass spectrometry analysis. a) Chromatogram of a sample of 1 % isoflurane in perfusate solution. The peaks at retention times 2.64 min and 3.20 min were identified as CO_2 and isoflurane, respectively, by mass spectral analysis. **b)** Mass spectra for isoflurane ($C_3H_2ClF_5O$; molecular weight = 184.49 g/mol) showed characteristic molecular and fragment ions: m/z 149 ($C_3H_2F_5O$ – Cl) and 116.9 (C_2HClF_3 – $OCHF_2$). **c)** Mass spectra for CO_2 showed the characteristic peak at m/z 44 (molecular weight = 44 g/mol).

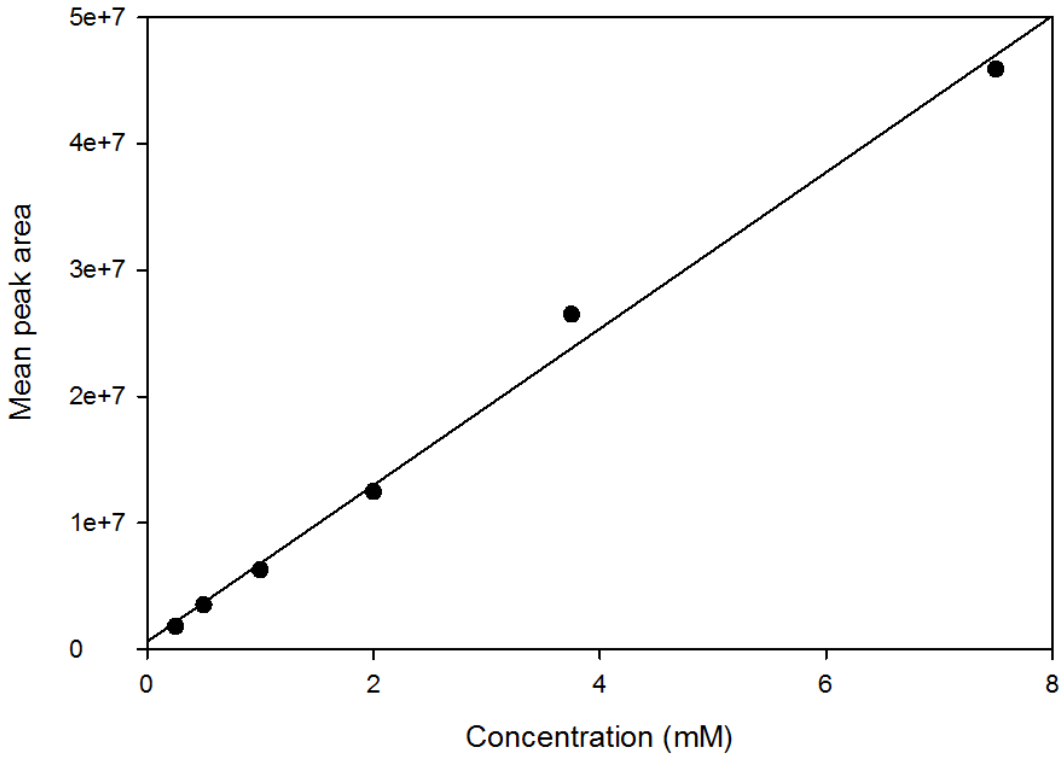

Figure 2.5. Standard curve for isoflurane based on GC-MS analysis of perfusate. A standard curve for isoflurane was generated from a saturated solution of isoflurane (15 mM; Scheller et al., 1997). Dilutions of the saturated solution (1:2, 1:4, 1:8, 1:16, 1:32, and 1:64) were analyzed by gas-chromatography-mass spectrometry. Mean peak area corresponds to the number of isoflurane molecules in solution. The standard curve follows a linear regression line described by the equation: $y = 6.2*10^6 x + 6.0*10^5$ ($R^2 = 0.994$).

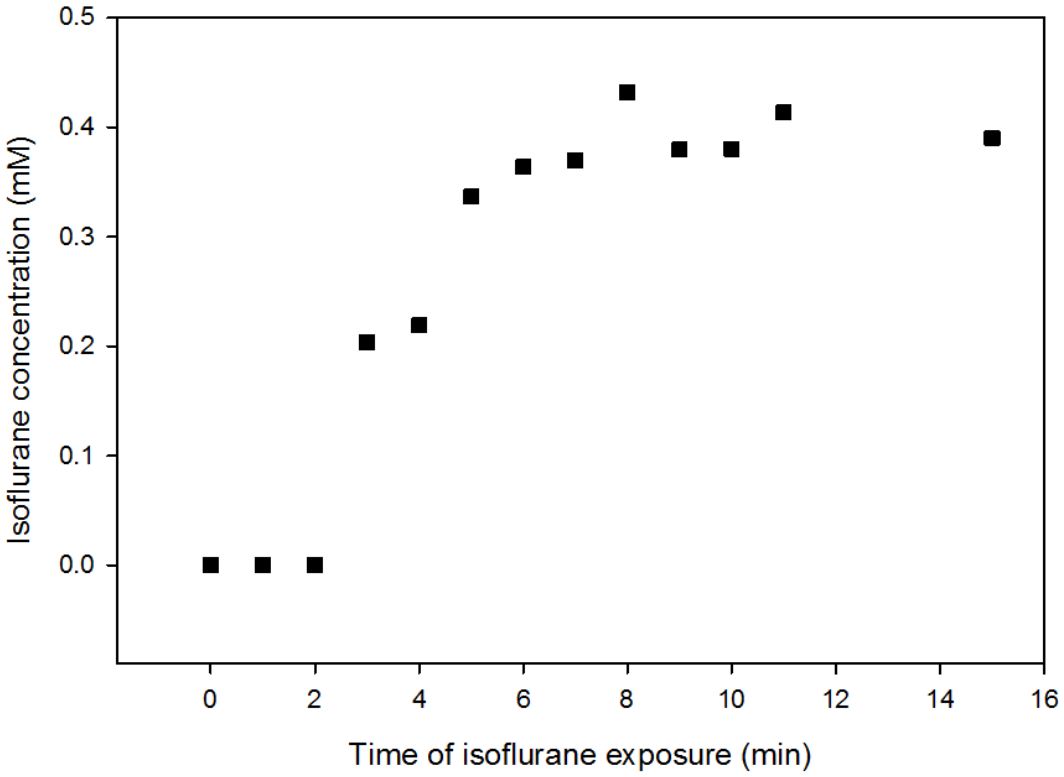

Figure 2.6. Isoflurane concentration in perfusate is equivalent to 1 volume percent. Gas chromatography - mass spectrometry analysis was performed on perfusate samples taken at each minute during a 10-min 1 % isoflurane exposure. The concentration of isoflurane in solution at each minute of the drug exposure was calculated from the standard curve equation: y = 6.2*10^6 x + 6.0*10^5 (R^2 = 0.994). At 10 min, the time point when all neuronal responses to isoflurane were recorded, the average equilibrium isoflurane concentration was 0.38 mM, which was equivalent to 1 volume percent (0.38 mM = average concentration from 6 to 10 min; 0.37 mM = 1 volume percent; Eilers et al., 1999). Additional samples were taken at 11 and 15 min to confirm that isoflurane concentrations were stable throughout neuronal recording periods.

Chapter 3

The response of CO_2-inhibited neurons to isoflurane: Evidence for a heterogeneous population of medullary raphé GABA neurons[2]

3.1 Abstract

Anesthetics are widely used in clinical and scientific practice, but mechanisms of anesthetic action remain largely undefined. Specifically, the influence of anesthetics on central chemoreceptors, neurons that sense changes in arterial CO_2/pH, remains a question that is relevant to understanding the depressed hypercapnic ventilatory response during anesthesia. Here, we investigated the influence of isoflurane on GABA neurons, which are proposed chemoreceptors in the medullary raphé, using the arterially perfused *in situ* brainstem preparation. We tested the hypothesis that 1 % isoflurane, approximating 1.15 MAC for isoflurane, enhances action potential discharge in medullary raphé GABA neurons and disrupts their chemosensitivity. We documented a heterogeneous population of CO_2-inhibited putative GABA neurons, represented by at least two distinct groups, which differed in their baseline firing frequencies and response to isoflurane. Furthermore, we found that isoflurane disrupted the chemosensitivity of these CO_2-inhibited putative GABA neurons. This study offers insight into the contribution of GABA neurons to the depressed ventilatory response that is characteristic of general anesthesia.

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[2] Johansen SL, Iceman KE, Richerson GB, and Harris MB. 2014. The response of CO_2-inhibited neurons to isoflurane: Evidence for a heterogeneous population of medullary raphé GABA neurons. In preparation for submission in the *British Journal of Anesthesiology*.

3.2 Introduction

The molecular mechanisms that give rise to the state of anesthesia are poorly understood, despite the widespread use of anesthetics in clinical and scientific practice. Several theories have been historically proposed to answer the critical question of how a wide array of anesthetic agents with diverse molecular structures produce the same state of anesthesia, characterized by immobility, hypnosis, analgesia, and amnesia. These theories of general anesthetic mechanisms of action have evolved from a unitary theory of non-specific anesthetic action, to a theory that anesthetics act on specific protein targets, and now to a multiple mechanism concept. It is now clear that anesthetics act at multiple anatomical sites and on multiple molecular targets to produce the state of anesthesia (Campagna et al., 2003; Grasshoff et al., 2005; Torri, 2010). The effect of anesthetics on a vast array of protein targets, including membrane receptors, ion channels, serum proteins, and intracellular signaling molecules (Rudolph and Antkowiak, 2004), is the current focus of research that seeks to relate molecular actions of anesthetics to behavioral states of anesthesia.

Virtually all areas in the central nervous system experience depressed neuronal responses during anesthesia. The current working hypothesis states that system-wide depression results from enhanced inhibitory postsynaptic activity through action on $GABA_A$ [receptor subtype for the inhibitory neurotransmitter γ-aminobutyric acid (GABA)] and glycine receptors and decreased excitatory postsynaptic activity through action on glutamate, nicotinic acetylcholine, and serotonin receptors (Franks and Lieb, 1994;

Campagna et al., 2003; Torri, 2010). The majority of research focuses on postsynaptic sites of anesthetic action, particularly $GABA_A$ receptors, which are the most abundant receptors for inhibitory neurotransmitters in the brain. General anesthetics increase the sensitivity of $GABA_A$ receptors to GABA at clinically relevant concentrations and prolong the $GABA_A$ receptor-mediated current in response to a pulse of GABA, thereby inhibiting postsynaptic neuronal excitability (Jones and Harrison, 1993). Many classes of both inhalational and intravenous anesthetics have been shown to prolong inhibitory post-synaptic currents (IPSCs) mediated by $GABA_A$ receptors (Krasowski and Harrison, 2000). This potentiation of the $GABA_A$ receptor-mediated current *in vitro* corresponds to the anesthetic potency measured by MAC-immobility (minimum alveolar concentration that prevents movement in response to noxious stimuli in 50 % of subjects) *in vivo* (Zimmerman et al., 1994). In humans, positron emission tomography scans using a ^{11}C-labeled benzodiazepine ligand, a $GABA_A$ receptor-sensitive probe, indicate that isoflurane dose-dependently enhances binding of the ligand to $GABA_A$ receptors, which may suggest that a conformational change of $GABA_A$ receptors occurs during isoflurane anesthesia in the human brain (Gyulai et al., 2001). These and many other studies provide strong evidence that $GABA_A$ receptors play a major role in mechanisms of anesthetic action.

The wide body of literature on $GABA_A$ receptors suggests that postsynaptic sites predominate in anesthetic mechanisms, but evidence is emerging that supports presynaptic sites as critical targets for anesthetics (Griffiths and Norman, 1993; Pocock

and Richards, 1993). Presynaptic sites of anesthetic action are implied from electrophysiological studies that compare the effect of presynaptic stimulation and direct application of neurotransmitter on postsynaptic potentials in the presence of anesthetics. For example, 1 MAC halothane reduced glutamatergic excitatory postsynaptic currents in mouse hippocampal slices by approximately 50 %, but halothane had no effect, even at concentrations approximating 5 MAC, on postsynaptic currents induced by glutamatergic agonists (Perouansky et al., 1995). Presynaptic sites are also implicated from studies that show that anesthetics affect neurotransmitter release. Although molecular mechanisms are not yet defined, the volatile anesthetic isoflurane has been shown to inhibit the mammalian neurotransmitter release in neurons (Herring et al., 2009), and this inhibition is thought to be the result of interaction with vesicle fusion proteins, SNARE and SNARE-associated proteins (Xie et al., 2013). Volatile anesthetics have also been shown to inhibit voltage-gated Na^+ channels (Herold and Hemmings, 2012), two-pore-domain background K^+ channels (TASK channels; Bayliss and Barrett, 2008), and voltage-gated Ca^{2+} channels (Orestes and Todorovic, 2010), which could affect membrane excitability, action potential conductance, and neurotransmitter release.

Given the working hypothesis that central nervous system depression during anesthesia involves both inhibition of excitatory transmission and enhancement of inhibitory transmission, many studies on presynaptic neurotransmitter release focus on glutamate versus GABA release. Isoflurane has been shown to selectively inhibit Na^+ channel-dependent 4-aminopyridine (4AP)-evoked release of glutamate compared to GABA

release (Westphalen et al., 2011; Westphalen et al., 2013). Volatile anesthetics were also shown to preferentially inhibit Ca^{2+}-dependent 4AP-evoked glutamate versus GABA release (Westphalen and Hemmings, 2006). These studies conclude that anesthetics inhibit glutamate release more potently than GABA release. The influence of anesthetics on GABA release is a subject of some controversy, as both inhibition and enhancement of GABA release in response to anesthetics are reported. Some studies report that anesthetics act on GABAergic presynaptic nerve terminals to decrease action potential dependent GABA release (Ogawa et al., 2011; Kotani and Akaike, 2012), while others conclude that anesthetics increase inhibitory postsynaptic current (IPSC) frequency and GABA release from presynaptic nerve terminals (Larsen et al., 1998; Murugaiah and Hemmings, 1998; Pittson et al., 2004). The current literature supports the conclusion that anesthetics act on GABA neurons presynaptically but, clearly, molecular mechanisms have yet to be resolved. Additionally, the question of how those molecular actions relate to the state of anesthesia has yet to be systematically investigated.

A component of the state of anesthesia that is particularly relevant in clinical settings is the influence of anesthetics on control of breathing, since respiratory depression during anesthesia is a well-known critical concern (Evers et al., 2006). Volatile halogenated anesthetics, such as isoflurane, affect both the chemical control of breathing, which responds to changes in the chemical composition of arterial blood (pH, PCO_2, PO_2), and the behavioral control of breathing, which responds to changes in behavioral states, such as arousal or stress (Dahan and Teppema, 2003). In humans, the ventilatory response to

decreased O_2 (hypoxia) and increased CO_2 (hypercapnia) are depressed by volatile anesthetics, including halothane (Dahan et al., 1994), isoflurane (Sollevi and Lindahl, 1995; van den Elsen et al., 1998), and sevoflurane (Sarton et al., 1996). The mechanism by which this depressed ventilatory response occurs, however, is not fully defined.

Chemoreceptors, cells that function to sense changes in CO_2/pH and stimulate respiratory network responses necessary to regain homeostasis, are thought to play a primary role in mechanisms that give rise to blunted ventilatory responses during anesthesia. The first studies on the effect of anesthetics on the ventilatory response in humans showed that low-dose anesthetics selectively impaired hypoxic and hypercapnic drive in peripheral chemoreceptors, but not in central chemoreceptors (Knill and Gelb, 1978; Knill and Clement, 1985). This conclusion has been corroborated by a number of more recent studies (Dahan et al., 1994; van den Elsen, 1995). These studies used subanesthetic concentrations (0.5 to 0.1 MAC) to select for the peripheral rather than central response. In a later study, van den Elsen et al. (1998) considered level of sedation of human subjects in addition to the anesthetic concentration, and suggested that at levels of sedation comparable with sleep volatile anesthetics (isoflurane, desflurane, and sevoflurane) depressed central and peripheral chemoreflexes. It is now understood that volatile anesthetics affect both peripheral and central chemoreflexes at clinical concentrations. For example, halothane has been shown to depress ventilatory drive by abolishing peripheral drive from peripheral chemoreceptors in the carotid bodies (Dahan et al., 1994) and by inhibiting central drive from major respiratory control networks, such

as the pre-Bötzinger complex (Koizumi et al., 2010). These prior studies indicate that central mechanisms of anesthetic action contribute to the blunted ventilatory response under anesthesia, but the direct influence of anesthetics on central chemoreceptors remains an important question.

The medullary raphé region in the brainstem is associated with homeostatic regulation and central respiratory control *in vivo*. Intrinsically chemosensitive neurons of two distinct phenotypes, including CO_2-stimulated serotonin (5-HT) and CO_2-inhibited γ-aminobutyric acid (GABA) synthesizing cells, have been identified in the medullary raphé *in vitro* (Richerson et al., 2001), and both serotonergic and GABAergic systems have been implicated in facilitation of the homeostatic response to hypercapnia/acidosis. Research using unanesthetized rat *in situ* perfused decerebrate brainstem preparations suggests these raphé neurons also function as central chemosensors in intact animals (Iceman et al., 2010; Iceman et al., 2013). As these cell types are known to be involved in central chemoreception, they may contribute to the depressed central chemoreflex that results in a blunted ventilatory response during anesthesia.

We have previously described the influence of isoflurane on 5-HT neurons, which are proposed to provide excitatory input in central respiratory control (Johansen et al., 2012; Massey et al., 2013). Here, we investigated the proposed inhibitory input in central respiratory control by documenting the effect of isoflurane on action potential discharge and chemosensitivity of GABA neurons. We tested the hypothesis that isoflurane

enhances action potential discharge of medullary raphé GABA neurons and disrupts their chemosensitivity using the arterially perfused *in situ* brainstem preparation. We demonstrated the influence of 1 % isoflurane on medullary raphé GABA neurons, a concentration that approximates 1.15 MAC (isoflurane concentration required for surgical anesthesia; Eger, 1981). Our present aim was to investigate the presynaptic influence of anesthetics on GABA neuron action potential discharge, which will add to the growing body of literature describing presynaptic mechanisms of anesthetic action. We also aimed to assess the effect of isoflurane on GABA neuron chemosensitivity, which may offer insight into the central mechanisms that contribute to the blunted ventilatory response to hypercapnia during anesthesia.

3.3 Methods

Experimental preparations: Animal care and experimental procedures followed guidelines set by the National Institutes of Health Office of Laboratory Animal Welfare and the United States Department of Agriculture Animal Welfare Act. All protocols were in accordance with the University of Alaska Fairbanks Institutional Animal Care and Use Committee guidelines. Juvenile male rats (50 – 150 g; Sprague-Dawley strain; Simonson Laboratories) were used to generate the perfused *in situ* brainstem preparation as previously described (Paton, 1996; Corcoran et al., 2013, Iceman et al., 2013). Briefly, animals were administered an intraperitoneal heparin sodium injection (0.5 mL, 1000 USP; Sagent Pharmaceuticals) to prevent formation of blood clots during surgery. Deep anesthesia was induced using isoflurane and assessed by cessation of spontaneous

breathing and absence of a withdrawal response to firm toe pinch. Anesthesia was discontinued as preparations were bisected sub-diaphragmatically and decerebrated rostral to the superior colliculi, and subsequent procedures were conducted in the absence of anesthesia. Preparations were immersed in chilled artificial cerebral spinal fluid (aCSF) for the remainder of the dissection, which was conducted to isolate the descending aorta.

Each preparation was placed in the recording chamber in a prone position. The descending aorta was cannulated retrogradely with a double-lumen catheter, and preparations were perfused with a solution at a temperature of 31 °C. The perfusate solution contained the following (in mM): 1.0 $MgSO_4$, 125 NaH_2PO_4, 4.0 KCl, 24 $NaHCO_3$, 115 NaCl, 10 D-Glucose, 2.0 $CaCl_2$, and 0.18 Ficoll. Baseline conditions, when the perfusate was equilibrated with 95 % O_2 – 5 % CO_2 (PCO_2 33 mmHg; pH=7.4), approximated normocapnic plasma *in vivo*. Levels of O_2 and CO_2 in the perfusate were controlled by equilibrating a perfusate reservoir with gas mixtures produced by a precision gas mixer (CWE GSM-3) and verified by a CO_2 analyzer (Applied Electrochemistry CD-3A). Following cannulation, perfusion pressure was gradually increased to 40-60 mmHg by regulating the speed of a peristaltic perfusion pump. The first 50 mL of perfusate that passed through the preparation was discarded to eliminate blood, and the remaining 450 mL of perfusate was recirculated. The neuro-muscular blocker gallamine triethidodide (60 mg/L, Sigma-Aldrich) was added to the perfusate to

eliminate movement. The occipital bone, surrounding musculature, and cerebellum were removed to expose the dorsal surface of the brainstem.

Extracellular recording and experimental treatments: Extracellular recordings of medullary raphé (r. magnus, r. obscurus, r. pallidus) neurons were made using pulled-glass capillary electrodes (15–20 MΩ) filled with biotinamide hydrobromide (5 % in 0.5 M sodium acetate, Life Technologies). The electrode was positioned along the brainstem midline (≤ 0.1 mm lateral) 0 to 3 mm rostral of obex and advanced through the brain tissue in 2 μm increments using a fine stepping motor (Burleigh Instruments Inchworm). Extracellular recordings were made with an intracellular amplifier (Axon Instruments Multiclamp 700B) in current clamp mode, with a high pass filter at 300 Hz and low pass filter at 1 kHz bessel with an high impedance headstage (Axon CV7B, Molecular Devices). Action potentials were digitized using Spike 2 (Cambridge Electronic Design Power 1401) and LabChart (AD Instruments) software and stored as computer data files.

Neuronal recordings were initiated under baseline conditions (95 % O_2 – 5 % CO_2; PCO_2 33 mmHg; pH=7.4). As stated above, these conditions approximate normocapnic plasma *in vivo*; however, due to the lack of hemoglobin, the solution hyperoxia (PO_2 ~ 600 mmHg) was necessary to maintain O_2 content sufficient to meet tissue metabolic demands, and this unavoidable hyperoxia was constant under all conditions. The normocapnic condition was followed by a hypercapnic challenge (91% O_2 – 9% CO_2; PCO_2 60 mmHg; pH 7.2; 5 min) to approximate conditions similar to those in plasma

during a 4 % increase in inspired CO_2. The preparation was then returned to normocapnic conditions for an additional 5 min. The 5-min exposure was demonstrated to be sufficient for brain tissue equilibration with the 91 % O_2 – 9 % CO_2 gas levels in the perfusate (Wilson et al., 2001).

Spontaneously active neurons were identified electrophysiologically and classified according to changes in firing frequency elicited by elevation of arterial CO_2 concentrations during hypercapnic challenges (increase, decrease, and no change in baseline firing frequency). We have previously shown that CO_2-inhibited medullary raphé neurons may be considered putative GABA neurons (Iceman et al., 2010). All CO_2-inhibited neurons were considered putative GABA neurons and were selected for further recording. CO_2-inhibited putative GABA neurons were administered isoflurane bubbled into the perfusate for 10 min (1, 1.5, or 2 % isoflurane in 95 % O_2 – 5 % CO_2; percent isoflurane refers to volume percent). For neurons that did not cease under isoflurane, the 5-min hypercapnic challenge was repeated in the presence of isoflurane (1, 1.5, or 2 % isoflurane; 91 % O_2 – 9 % CO_2). Hypercapnic responses under isoflurane could not be assessed for neurons that ceased firing in response to 1% isoflurane. Upon return to baseline conditions (0 % isoflurane; 95 % O_2 – 5 % CO_2), neurons were allowed to recover toward baseline firing frequency. Recovery times ranged from 10-30 min, depending on the individual neuronal characteristics and assays performed.

Juxtacellular labeling: Subsets of recorded neurons were individually filled with biotinamide using the juxtacellular labeling method (Pinault, 1996; Winkler et al., 2006). Extracellular recordings were conducted in current clamp mode (Axon Multiclamp 700B) to allow current to be injected through the electrode while action potentials were monitored. Positive current pulses were applied through the electrode (400-ms duration) and gradually increased from 0-15 mV (0.5 mV steps; Grass Stimulator S44B) until the cell was entrained to fire simultaneously with the applied stimulus. Injected current, ejection of biotinamide, and cellular firing entrainment caused uptake of the biotinamide marker by the recorded cell. Entrainment was maintained for at least 30 s. After termination of entrainment, the biotinamide was allowed to disperse for 30 min. Rats were then perfused through the descending aorta with fixative [4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer saline (PBS); pH 7.4; Sigma-Aldrich]. Brainstems were then removed and stored in fixative prior to sectioning. Coronal sections (60 μm) were cut through the medulla (Vibratome 1000Plus), and sections were processed free-floating.

Immunohistochemistry: Coronal tissue sections were blocked and permeabilized in blocking buffer [Blocking buffer: 5 % normal goat serum (NGS in 0.1 % sodium axide; Caltag Spec. No. L10000, Lot: 384935A) and 0.3 % Triton X-100 in 0.1 M PBS] for 1 hr at 22 °C. Sections were incubated with a streptavidin-Alexa 546 conjugate (Life Technologies #S-11225; 1:500 dilution in PBS with 5 % NGS) for 2 hr at 22 °C to reveal biotinamide introduced into single neurons by juxtacellular labeling. To identify GABAergic cells, we used an antibody for the GABA-synthesizing enzyme glutamate

decarboxylase (GAD-67). Following incubation with mouse monoclonal anti-GAD67 (Millipore MAB5406), sections were incubated with goat anti-mouse Alexa 488 (Life Technologies #A11029) to label GAD67-immunoreactive cells. Sections were air-dried for 30 min, mounted with Vectashield (Vector Labs), and coverslipped. Low-magnification (10x) images were used to determine the location of biotinamide-labeled cells in relation to anatomical landmarks. Local biotinamide and GABA fluorescence identified colocalization of GABAergic markers in biotinamide-labeled neurons. Fluorophores were individually excited and emission spectra were collected separately to minimize interference using a Zeiss LSM510 confocal microscope: biotinamide-labeled neuron, Alexa 546, 543 nm laser, filter BP 560-615; anti-GAD67, Alexa 488, 488 nm laser, filter BP505-530. Images were constructed as 40x projections of z-stacks.

Data analysis: We discriminated extracellularly recorded individual unit activity using computer spike sorting software (Spike Histogram, AD Instruments). Stable 1-2 min periods of individual unit firing were analyzed before and during hypercapnic challenge to provide a mean value for unit firing frequency (spikes/s) and interspike interval (ms). Subset analysis was used to classify neurons as chemo-stimulated or chemo-inhibited where relative frequencies changed by greater than 20 %, and chemo-insensitive where relative frequencies changed by less than 20 % (Wang et al., 2001). For cells identified as CO_2-inhibited putative GABA neurons based on a minimum 20 % decrease in firing frequency during hypercapnic challenge, between groups comparison of the change in absolute firing frequency from normocapnia (5 % CO_2) to hypercapnia (9 % CO_2) and

recovery (5 % CO_2) was determined using one-way repeated-measures ANOVA. Post-hoc tests used Holm-Sidak pairwise comparison (SigmaPlot).

Considering all CO_2-inhibited neurons as a single group (n=19), between groups comparison of baseline firing frequency with firing frequency during 1 % isoflurane treatment was determined using a paired *t*-test. However, this analysis of the entire population of CO_2-inhibited putative GABA neurons did not reflect the occurrence of neurons that increased firing in response to 1 % isoflurane. Neurons were subsequently analyzed by percent change from baseline during isoflurane, to reveal a bimodal distribution represented by two groups: 1) “slow firing” cells that increased firing during isoflurane and 2) “fast firing” cells that decreased or ceased firing during isoflurane. Between groups comparison of the mean percent change from baseline of "slow firing" and "fast firing" CO_2-inhibited neurons was determined by *t*-test. Between groups comparison of mean baseline firing frequencies of "slow firing" and "fast firing" CO_2-inhibited neurons was determined by one-tailed *t*-test. The one-tailed *t*-test was used because the distribution of firing frequencies was truncated at zero, corresponding to a neuron that is not firing (0 Hz) and, therefore, the distribution was not normal and a two-tailed *t*-test was not appropriate for analysis. The between groups comparison of mean baseline firing frequencies was also determined by a *t*-test on ranks by the same justification of a distribution that is truncated at zero. Throughout the text, values are expressed as means ± standard error of the mean. The criteria for statistical significance was $P < 0.05$, and instances of $P < 0.01$ and $P < 0.001$ are also noted.

3.4 Results

CO_2-inhibited medullary raphé neurons are GABA-synthesizing neurons. Medullary raphé neurons that decreased in firing frequency by at least 20 % from baseline during hypercapnic challenge (9 % CO_2; pH 7.2; 5 min) were categorized as "CO_2-inhibited" neurons, and these CO_2-inhibited neurons were identified as GABA-synthesizing neurons. Two representative cells are shown in Figure 3.1. Both neurons were inhibited during hypercapnic challenge and returned toward baseline firing rates upon return to normocapnia. The difference in baseline firing frequencies (0.27 Hz compared to 8.95 Hz) and patterns (tonic compared to bursting) between these two cells demonstrates the heterogeneity of baseline firing characteristics that we observe in medullary raphé CO_2-inhibited cells (Fig. 3.1a and 3.1c). Both cells were confirmed GABAergic by colocalization of markers of GABA-synthesis in biotinamide-labeled neurons (Fig. 3.1b and 3.1d).

CO_2-inhibited medullary raphé putative GABA neurons display heterogeneous responses to isoflurane. CO_2-inhibited putative GABA neurons exhibited two different responses to 1 % isoflurane. Isoflurane stimulated action potential discharge in a subset of CO_2-inhibited neurons and inhibited action potential discharge in a different subset of CO_2-inhibited neurons. A representative cell for the subset of isoflurane-stimulated CO_2-inhibited neurons is shown in Figure 3.2a-b. In this case, isoflurane stimulated action potential discharge by 1,622 %. A different cell representing the response of the subset of isoflurane-inhibited CO_2-inhibted neurons is shown in Figure 3.2c-d. In this case,

isoflurane caused action potential discharge to cease. In both cases, the cells recovered toward baseline firing upon washout of isoflurane.

Consideration of the total population of medullary raphé CO_2-inhibited putative GABA neurons indicates inhibition by isoflurane, but this response to isoflurane is not reflective of all neuron responses. Considering all CO_2-inhibited neurons as a single group (n=19), it appears that on average, 1 % isoflurane inhibited action potential discharge in the population as a whole. The mean firing frequency at baseline (2.14 ± 0.57 Hz) was significantly different from the mean firing frequency during 1 % isoflurane treatment (0.65 ± 0.26 Hz), as shown in Figure 3.3 (*$P < 0.05$). However, this analysis of the entire population of CO_2-inhibited putative GABA neurons did not reflect the occurrence of neurons that increased firing in response to 1 % isoflurane (see Fig. 3.2a-b). Consideration of the change in baseline firing frequency in response to 1 % isoflurane for each individual cell (n=19) indicated a significant degree of variability between neurons, both in terms of their absolute firing frequencies at baseline and responses to 1 % isoflurane (Fig. 3.4). The majority of neurons decreased or ceased firing during isoflurane treatment (n=14), but a subset of neurons increased firing in response to isoflurane (n=5).

Individual responses of medullary raphé CO_2-inhibited putative GABA neurons to 1 % isoflurane treatment are characterized by a bimodal distribution. The percent increase from baseline during 1 % isoflurane treatment revealed a bimodal distribution

represented by two groups: CO_2-inhibited neurons that increased firing in response to isoflurane and CO_2-inhibited neurons that decreased or ceased firing during isoflurane treatment (Fig. 3.5). Neurons that had a percent change from baseline greater than zero (n=5) increased firing in response to isoflurane. Neurons that had a percent change from baseline less than zero (n=14) decreased firing in response to isoflurane. Not all lines are apparent because neurons that ceased firing have overlapping lines and data points (n=10; 0 to -100 % on the y-axis).

The medullary raphé population of CO_2-inhibited putative GABA neurons is heterogeneous. The population of CO_2-inhibited putative GABA neurons is heterogeneous and represented by at least two distinct groups, which differ in their mean baseline firing frequencies and responses to isoflurane. The CO_2-inhibited neurons that increased firing in response to isoflurane were characterized as “slow firing” neurons with a mean baseline firing frequency of 0.50 ± 0.38 Hz, and the CO_2-inhibited neurons that decreased or ceased firing in response to isoflurane were characterized as “fast-firing” neurons with a mean baseline firing frequency of 2.72 ± 0.70 Hz (Fig. 3.6). Comparison of baseline firing frequencies of "slow firing" and "fast firing" CO_2-inhibited neurons indicated a significant difference between means (*one-tailed $P < 0.05$). Comparison of the percent change from baseline of "slow firing" (mean percent increase = 1043.86 ± 668.70 %) and "fast firing" CO_2-inhibited neurons (mean percent decrease = -85.82 ± 7.07 %) indicated a significant difference between means (**$P < 0.01$). In summary, the two groups of CO_2-inhibited putative GABA neurons observed in the

medullary raphé region are significantly different in terms of their mean baseline firing frequency and mean percent change from baseline in response to 1 % isoflurane.

A subset of medullary raphé neurons are CO_2-inhibited. All cells identified as CO_2-inhibited demonstrated a minimum 20 % decrease from baseline firing frequency during hypercapnic challenge (n=19; Fig. 3.7). Between groups comparison of the change in absolute firing frequency from normocapnia (5 % CO_2; pH 7.4) to hypercapnia (9 % CO_2; pH 7.2) and recovery (5 % CO_2; pH 7.4) indicated a significant difference between normocapnia and hypercapnia (***$P < 0.001$) and between hypercapnia and recovery (*$P < 0.05$). There was not a significant difference between firing frequencies during normocapnia and recovery periods ($P = 0.095$).

Isoflurane disrupts the chemosensitivity of medullary raphé CO_2-inhibited putative GABA neurons. CO_2-inhibited neurons that were stimulated by 1 % isoflurane did not respond to the hypercapnic challenge given during isoflurane treatment (9 % CO_2 in solution with 1 % isoflurane; pH 7.2; 5 min). A representative cell is shown in Figure 3.8. Considering all "slow-firing" isoflurane-stimulated neurons that received a hypercapnic challenge during isoflurane (n=5), there was not a significant difference in absolute firing frequency between normocapnia and hypercapnia during 1 % isoflurane treatment (Fig. 3.9; $P = 0.510$). Only those neurons that increased firing in response to isoflurane were included in analysis of chemosensitivity under isoflurane because hypercapnic responses could not be assessed in "fast-firing" neurons that ceased firing with isoflurane.

3.5 Discussion

Here, we present the first evidence of heterogeneity in the presynaptic response to isoflurane of medullary raphé CO_2-inhibited putative GABA neurons. We found that CO_2-inhibited cells responded to isoflurane in one of two distinct ways: action potential discharge of these cells either dramatically increased or significantly decreased, to the point of ceasing in the majority of cases, in response to 1 % isoflurane. Comparing the baseline firing characteristics of cells that were stimulated to cells that were inhibited, we found a significant difference in baseline firing rates. Isoflurane-stimulated neurons fired slowly at baseline (0.50 ± 0.38 Hz) compared to isoflurane-inhibited neurons (2.72 ± 0.70 Hz). Our results indicate a heterogeneous population of medullary raphé CO_2-inhibited putative GABA neurons represented by at least two distinct groups, which differ in their response to isoflurane and baseline firing frequency: 1) "slow-firing" CO_2-inhibited putative GABA neurons that are stimulated by isoflurane; and 2) "fast-firing" CO_2-inhibited putative GABA neurons that are inhibited by isoflurane.

It is now widely accepted that central depression during anesthesia occurs as a result of enhanced inhibitory neurotransmission and decreased excitatory neurotransmission (Franks and Lieb, 1994; Campagna et al., 2003; Torri, 2010). The majority of studies suggest that the enhanced inhibitory neurotransmission during anesthesia is due to potentiation of inhibitory currents through postsynaptic anesthetic action, notably on $GABA_A$ receptors, but a growing number of studies suggest that presynaptic sites are also critical components of anesthetic mechanisms (Griffiths and Norman, 1993; Pocock and

Richards, 1993). Here, we document a presynaptic response to anesthetic by recording action potentials before and after isoflurane treatment. Our observation of a population of CO_2-inhibited putative GABA neurons that are stimulated by isoflurane is consistent with the current working hypothesis of enhanced inhibitory neurotransmission during anesthesia. We postulate that the population of CO_2-inhibited putative GABA neurons that increased firing in response to isoflurane may contribute to central inhibition characteristic of general anesthesia. We did not directly measure neurotransmitter release, so this interpretation is based on the assumption that increased firing frequency results in increased action potential dependent release of GABA. The population of CO_2-inhibited putative GABA neurons that decreased or ceased firing in response to isoflurane, however, does not fit with the working hypothesis of enhanced inhibitory neurotransmission during anesthesia, as we would expect removal of inhibitory inputs to result in increased neural activity. The contribution of this response to the state of anesthesia remains a pertinent question.

Our identification of two opposing responses to isoflurane in CO_2-inhibited putative GABA neurons may be relevant in consideration of the current controversy surrounding anesthetic effects on GABA release, wherein some studies conclude that anesthetics stimulate release and others report that anesthetics inhibit GABA release. In the CA1 region of rat hippocampal slices, general anesthetics caused an increase in IPSC frequency, which was attributed to a presynaptic mechanism (Pittson et al., 2004). These findings corroborated earlier studies showing that both inhalational and intravenous

anesthetics increased IPSC frequency and calcium-dependent GABA release from nerve terminals in rat cerebral cortex (Larsen et al., 1998; Murugaiah and Hemmings, 1998). However, other studies indicate that anesthetics depress GABA release (Kotani and Akaike, 2012). Volatile anesthetics, including isoflurane, have been shown to decrease action potential dependent GABA release in synaptic boutons isolated from rat hippocampal CA1 neurons (Ogawa et al., 2011). Our observations of both isoflurane-inhibited and isoflurane-stimulated CO_2-inhibited putative GABA neurons are consistent with the literature that reports both inhibition and enhancement of GABA release. Collectively, these data suggest a heterogeneous response of GABA neurons to anesthetics. It is likely that whether GABA release is stimulated or inhibited by anesthetics is specific to the anesthetic agent, anatomical location, channel composition, and network inputs, and that these multiple sites and multiple effects are necessary for inducing the complex state of anesthesia.

To interpret the responses of CO_2-inhibited putative GABA neurons to isoflurane within this multi-site theory, and to begin characterizing the two populations of "slow-firing" isoflurane-stimulated neurons and "fast-firing isoflurane-inhibited neurons, we need to investigate molecular mechanisms underlying presynaptic enhancement or inhibition of action potential discharge in GABA neurons. A number of membrane channels are potential anesthetic targets. Convincing evidence exists to suggest that volatile anesthetics exert presynaptic effects on voltage-gated Na^+ channels (Herold and Hemmings, 2012), two-pore-domain background K^+ channels (TASK channels; Bayliss

and Barrett, 2008), and voltage-gated Ca^{2+} channels (Orestes and Todorovic, 2010). Volatile anesthetics have been shown to inhibit neurotransmission through blockade of Na^{+} channels leading to depression of action potential conductance (Ouyang and Hemmings, 2005; Wu et al., 2004), potentiation of background K^{+} currents leading to membrane hyperpolarization (Patel et al., 1999; Sirois et al., 2000), and inhibition of voltage-gated Ca^{2+} currents leading to decreased neurotransmitter release (Study, 1994; Nikonorov et al., 1998; Kamatchi et al., 1999). These and other presynaptic interactions are important to consider as potential underlying mechanisms for the population of putative GABA neurons that ceased firing during isoflurane treatment.

We postulate that the response of neurons that ceased firing during isoflurane may have been due to potentiation of the background K^{+} channels by isoflurane. The two-pore-domain channel subtypes TASK-1 and TASK-3 are highly expressed in the raphé nuclei compared to other brain regions (Talley et al., 2001). Binding of isoflurane to TASK channels on GABA neurons would hyperpolarize these neurons and inhibit action potential discharge during isoflurane treatment. Inhibition of action potential conductance through Na^{+} channel blockade may also be a contributing factor. Further research should explore these and other potential molecular mechanisms that give rise to isoflurane's inhibition of action potential discharge in GABA neurons.

With regards to the CO_2-inhibited putative GABA neurons that exhibited an increase in action potential discharge during isoflurane treatment, we postulate that the underlying

molecular mechanism may be related to the ionic mechanism that was previously described for retrotrapezoid nucleus (RTN) neurons that are stimulated by isoflurane (Lazarenko et al., 2010). In chemosensitive, Phox2b-expressing RTN neurons, the enhanced action potential discharge during isoflurane treatment was attributed to inhibition of a background K^+ current with properties similar to TWIK-related halothane-inhibited K^+ channels (THIK-1 channels). Although most subfamilies of the two-pore-domain K^+ channels, such as TASK-1 and TASK-3, show an increase in outward potassium current when exposed to inhalational anesthetics (Patel et al., 1999), the THIK-1 channels are inhibited by inhalational anesthetics (Rajan et al., 2001), and inhibition of the outward K^+ current leads to membrane depolarization and increased neuronal excitability. We postulate that the enhanced action potential discharge in CO_2-inhibited putative GABA neurons may be due to the inhibition of a background K^+ current through THIK-1 channels or other unidentified channels with similar properties. Regardless of specific mechanism or channel type, enhanced action potential discharge in CO_2-inhibited putative GABA neurons was accompanied by an disrupted response to hypercapnia during isoflurane treatment. We found that isoflurane-stimulated cells no longer exhibited a hypercapnic response during isoflurane treatment. The action potential discharge of these isoflurane-stimulated cells either did not change or continued increasing throughout the hypercapnic challenge with isoflurane. Although GABA neurons have been shown to be CO_2-inhibited *in vitro* and *in situ* (Richerson et al., 2001, Iceman et al., 2010; Iceman et al., 2013), the mechanism controlling GABA neuron chemosensitivity remains to be determined. Also unknown is whether the mechanism that

conferred enhanced action potential discharge is also responsible for disruption of the chemosensitive response, or if those effects were independent of each other.

The disrupted chemosensitive response that we observed in CO_2-inhibited isoflurane-stimulated putative GABA neurons may contribute to the decreased ventilatory response that is characteristic of general anesthesia. While the inhibitory effect of sub-anesthetic concentrations of volatile anesthetics has been abundantly documented in peripheral chemoreceptors (Knill and Gelb, 1978; Knill and Clement, 1985; Dahan et al., 1994; van den Elsen, 1998), the effect of volatile anesthetics on central chemoreceptors has not been completely defined. The volatile anesthetic halothane has been shown to depress ventilatory drive in part by inhibiting central drive from major respiratory control networks, such as the pre-Bötzinger complex (Koizumi et al., 2010). Our results documenting that isoflurane inhibits chemosensitivity in CO_2-inhibited putative GABA neurons add to the literature describing an inhibitory effect of volatile anesthetics on neurons involved in the central chemoreflex. This inhibition likely contributes to the respiratory depression characteristic of general anesthesia (Evers et al., 2006).

The present study was limited by the inability to conclusively identify all CO_2-inhibited neurons that were used for electrophysiological recordings as GABAergic. The juxtacellular labeling method used to fill neurons with biotinamide for immunohistochemical identification is by nature a low-yield process. However, our identification of a subset of CO_2-inhibited cells as GABA-synthesizing neurons support

prior *in vitro* and *in situ* studies that document a population of medullary raphé GABA neurons that are inhibited during hypercapnia (Corcoran et al., 2008; Iceman et al., 2010). We recognize that it is certainly probable that not all GABA neurons in the medullary raphé are CO_2-inhibited, and due to the inherent difficulty of the juxtacellular labeling method, we are not able to label all recorded neurons; however, we have yet to document a CO_2-inhibited cell that was not identified as a GABA neuron by colocalization of markers of GABAergic synthesis in the biotinamide-labeled neuron.

Future studies should focus on fully characterizing the two populations of GABA neurons. We found significantly fewer of the "slow-firing" isoflurane-stimulated cells (n=5) compared to "fast-firing" isoflurane-inhibited cells (n=14), but the relative proportions of these two populations in the medullary raphé is not known. Furthermore, it is not clear what properties of isoflurane-stimulated cells cause them to fire significantly slower at baseline compared to isoflurane-inhibited cells. We postulate that the mechanism underlying the response of "fast-firing" isoflurane-inhibited cells may be attributed to potentiation of the background K^+ current through TASK channels, and we further hypothesize that the mechanism responsible for the response of "slow-firing" isoflurane-stimulated cells could be attributed to inhibition of the background K^+ current through THIK-1 channels, but these hypotheses need to be investigated. Furthermore, the contribution of each population of isoflurane-stimulated and isoflurane-inhibited putative GABA cells to the state of anesthesia is a remaining question. In terms of the chemosensitive response, we postulate that the disrupted chemosensitivity of GABA

neurons by isoflurane contributes to the respiratory depression characteristic of general anesthesia, but this has not been confirmed. Further investigation of the molecular action of isoflurane on GABA neurons, and how this molecular action relates to the state of anesthesia and ventilatory depression during anesthesia, is required.

In summary, we demonstrate a heterogeneous population of medullary raphé CO_2-inhibited putative GABA neurons that is represented by at least two distinct groups, which differ in their baseline firing frequencies and response to isoflurane. This study documents how isoflurane affects action potential discharge and chemosensitivity in GABA neurons and contributes to literature that describes a presynaptic site of anesthetic action. Elucidating these molecular actions may offer insight into the contribution of GABA neurons to the respiratory depression characteristic of general anesthesia.

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3.7 Figures

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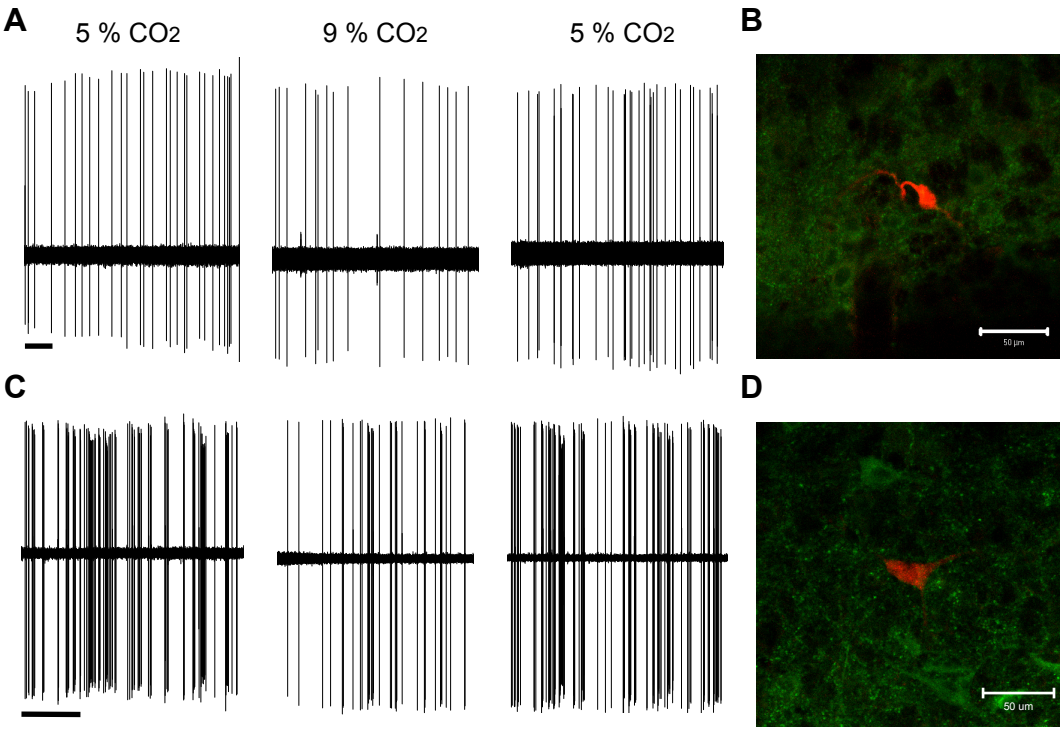

Figure 3.1. CO_2-inhibited medullary raphé neurons are GABAergic.
a) A neuron firing at 0.27 Hz at baseline decreased firing by 68.8 % during hypercapnic challenge (9 % CO_2; pH 7.2; 5 min) and recovered toward baseline firing upon return to normocapnia. Scale bar = 5 s. **b)** Fluorescent markers of GABA synthesis [glutamate decarboxylase (GAD67); green] and biotinamide (red) colocalize (yellow) to indicate the recorded neuron was GABAergic. Scale bar = 50 µm. **c)** A different neuron firing at 8.95 Hz at baseline decreased firing by 43.4 % during hypercapnic challenge and recovered toward baseline firing upon return to normocapnia. Scale bar = 5 s. **d)** Fluorescent markers of GABA synthesis (GAD67; green) and biotinamide (red) colocalize (yellow) to indicate the recorded neuron was GABAergic. Scale bar = 50 µm. Note different time scales for panels a and c.

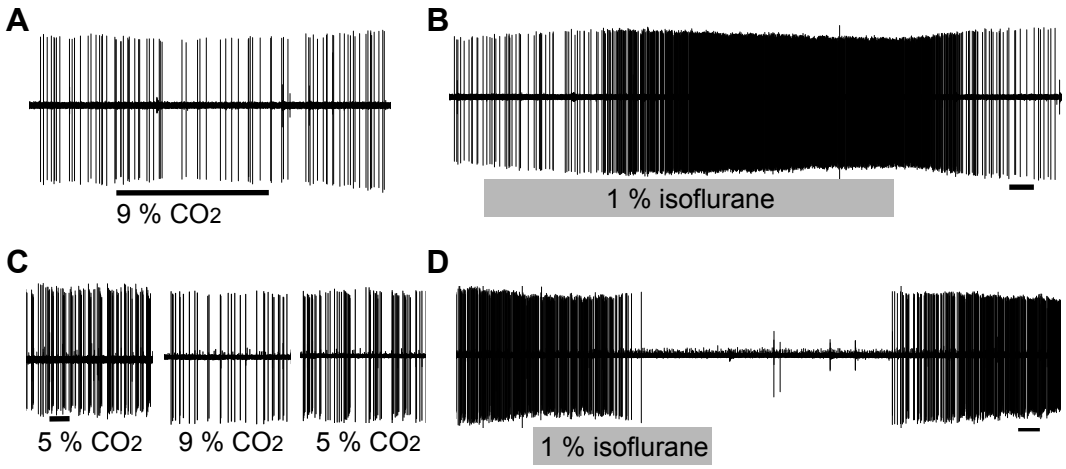

Figure 3.2. CO_2-inhibited putative GABA neurons display heterogeneous responses to isoflurane. a) A neuron firing at 0.13 Hz at baseline was inhibited during hypercapnic challenge (9 % CO_2; pH 7.2; 5 min) and recovered toward baseline firing upon return to normocapnia. **b)** The same neuron increased firing by 1,622 % during treatment with 1 % isoflurane. Firing frequency returned to baseline upon washout. Scale bar = 1 min. **c)** A different neuron firing at 2.92 Hz at baseline was inhibited during hypercapnic challenge (9 % CO_2; pH 7.2) and recovered toward baseline firing upon return to normocapnic conditions. Scale bar = 10 sec. **d)** Continued recording from the same neuron indicated the neuron ceased firing during treatment with 1 % isoflurane. Firing frequency returned to baseline firing upon washout. Scale bar = 1 min. Note different time scales for panels a-d.

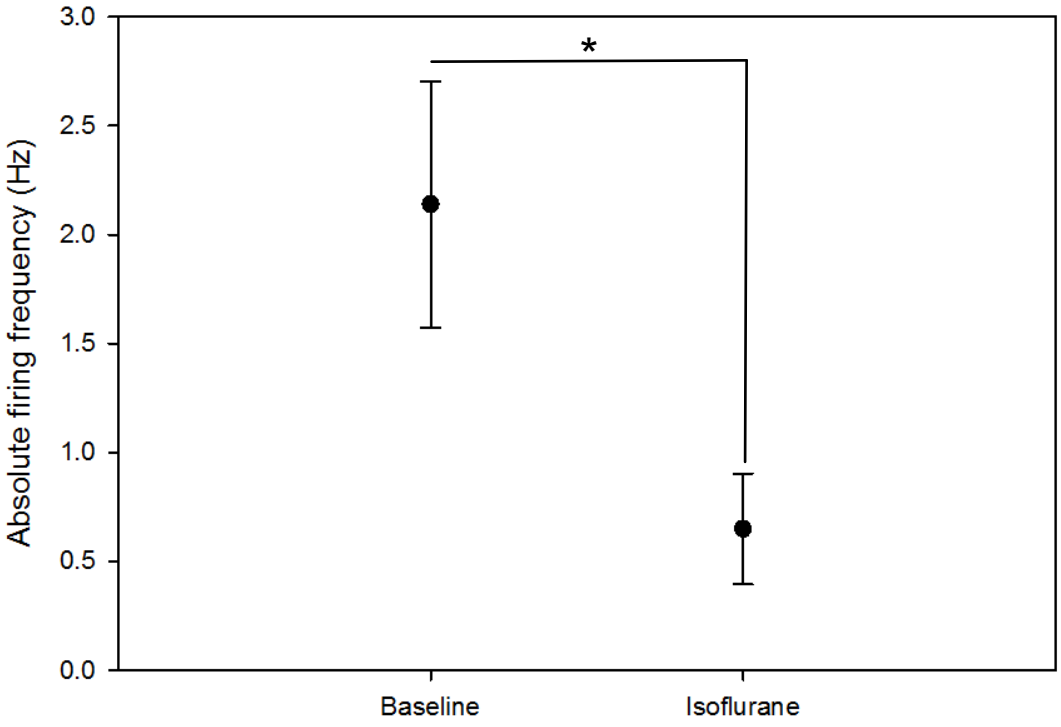

Figure 3.3. Consideration of the total population of CO_2-inhibited putative GABA neurons reflects population-wide inhibition by isoflurane. Considering all CO_2-inhibited neurons as a single group (n=19), the mean baseline firing frequency (2.14 ± 0.57 Hz) decreased during treatment with 1 % isoflurane (0.65 ± 0.26 Hz). Between groups comparison of baseline firing frequency to firing frequency during 1 % isoflurane treatment, determined by paired *t*-test, indicated a significant difference between means (*$P < 0.05$). This analysis of the entire population of CO_2-inhibited putative GABA neurons did not reflect the occurrence of neurons that increased firing in response to 1 % isoflurane (see Fig. 3.2a-b).

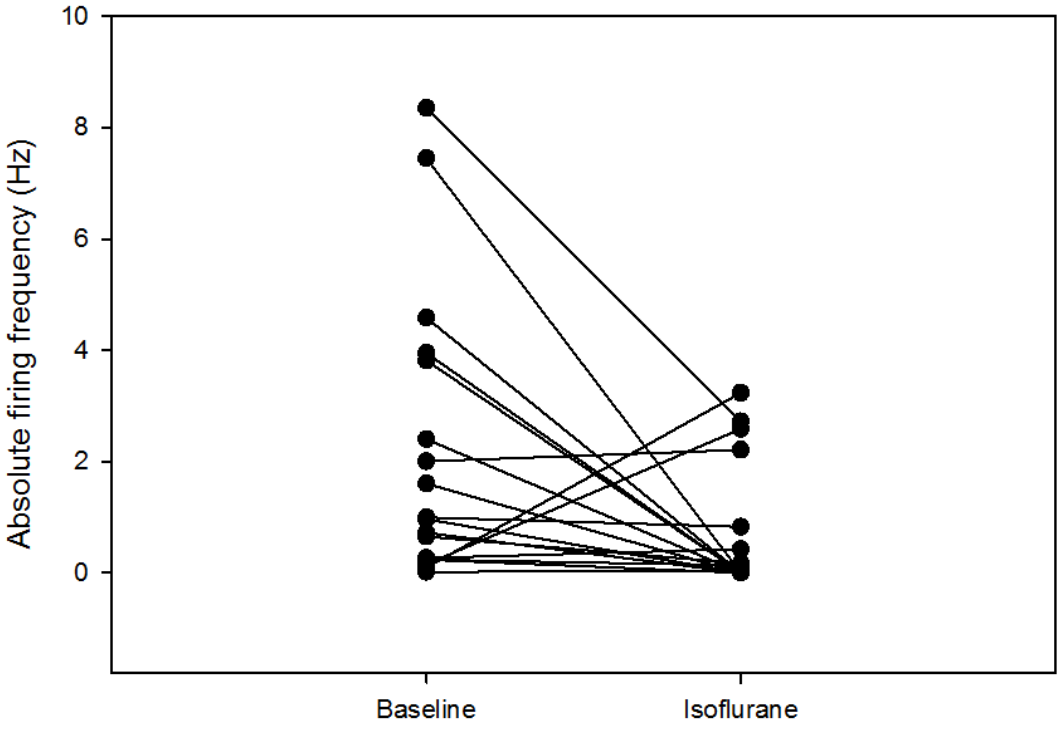

Figure 3.4. Individual responses of CO_2-inhibited putative GABA neurons to 1 % isoflurane treatment. The change in absolute firing frequency from baseline in response to 1 % isoflurane for each individual CO_2-inhibited putative GABA neuron (n=19) indicated a significant degree of variability between neurons, both in baseline firing frequency and response to isoflurane. The majority of neurons decreased or ceased firing during isoflurane treatment, but a subset of neurons increased firing in response to isoflurane.

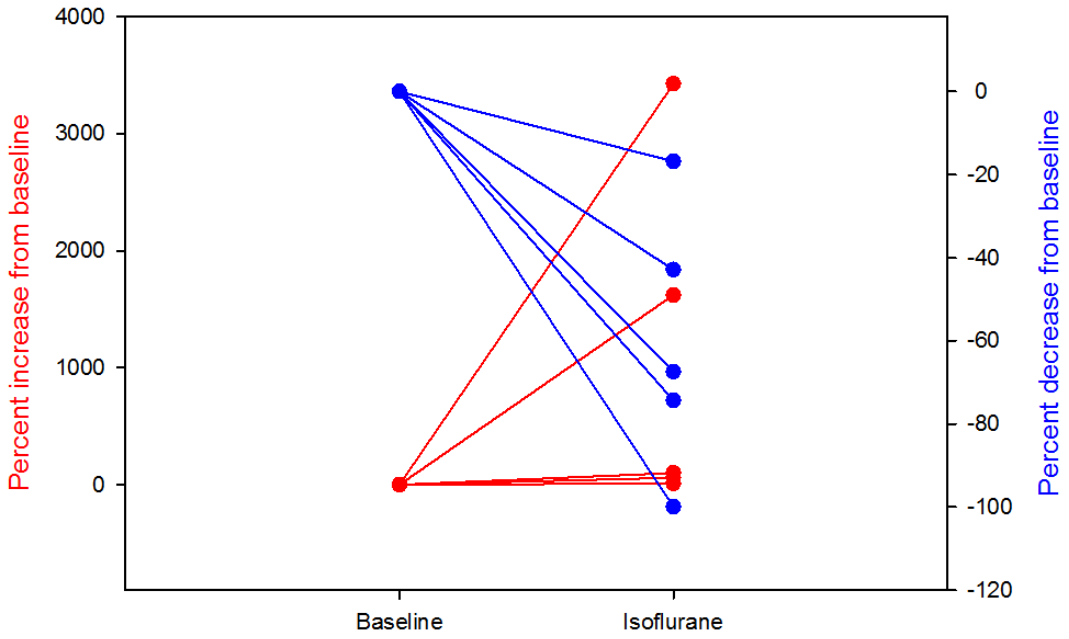

Figure 3.5. Individual responses of CO_2-inhibited putative GABA neurons to 1 % isoflurane treatment are characterized by a bimodal distribution. The percent increase from baseline during 1 % isoflurane treatment revealed a bimodal distribution represented by two groups: CO_2-inhibited neurons that increased firing in response to isoflurane and CO_2-inhibited neurons that decreased or ceased firing during isoflurane treatment. Neurons that had a percent change from baseline greater than zero (n=5, shown in red) increased firing in response to isoflurane. Neurons that had a percent change from baseline less than zero (n=14, shown in blue) decreased firing in response to isoflurane. Not all lines are apparent because neurons that ceased firing have overlapping lines and data points (n=10; 0 to -100 % on the y-axis).

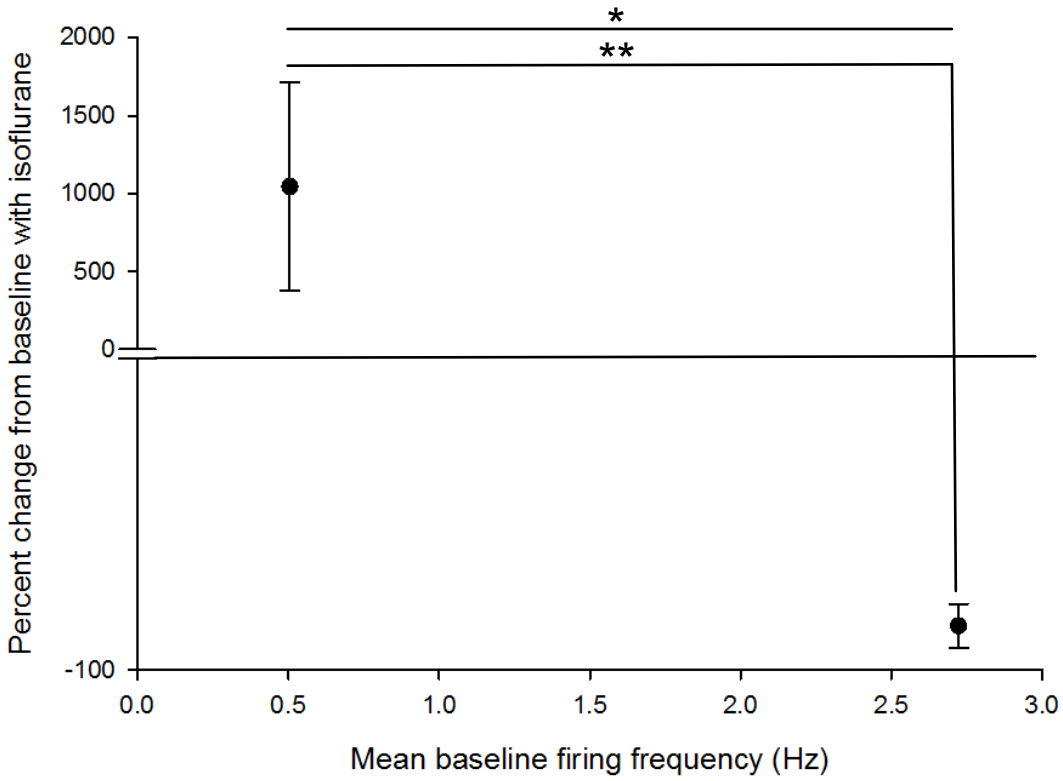

Figure 3.6. Characterization of two distinct populations of CO_2-inhibited putative GABA neurons. The population of CO_2-inhibited putative GABA neurons is heterogenous. The "slow-firing" (0.50 ± 0.38 Hz) CO_2-inhibited neurons increased firing in response to isoflurane. The "fast-firing" (2.72 ± 0.70 Hz) CO_2-inhibited neurons decreased or ceased firing in response to isoflurane. Comparison of baseline firing frequencies of "slow firing" and "fast firing" CO_2-inhibited neurons indicated a significant difference between means, determined by *t*-test (*one-tailed $P < 0.05$). Comparison of the percent change from baseline of "slow firing" (mean percent increase = 1043.86 ± 668.70 %) and "fast firing" CO_2-inhibited neurons (mean percent decrease = -85.82 ± 7.07 %) indicated a significant difference between means, determined by *t*-test (**$P < 0.01$).

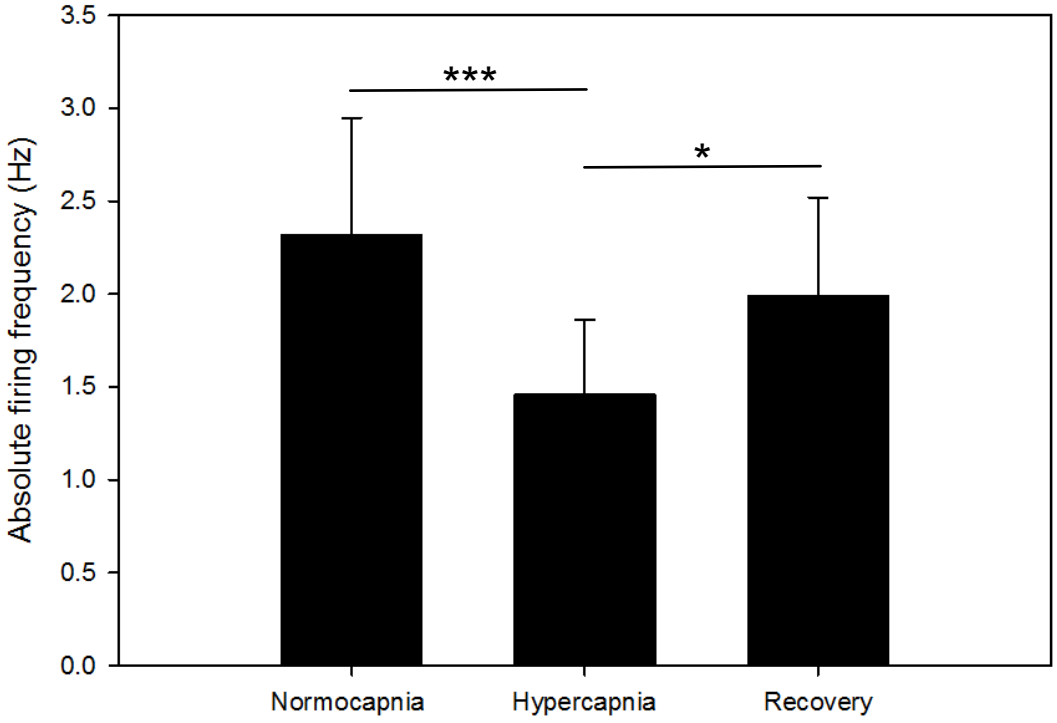

Figure 3.7. A subset of medullary raphé neurons are CO_2-inhibited. Cells identified as CO_2-inhibited (n=19) demonstrated a minimum 20 % decrease from baseline firing frequency during hypercapnic challenge. Between groups comparison of the change in absolute firing frequency from normocapnia (5 % CO_2; pH 7.4) to hypercapnia (9 % CO_2; pH 7.2) and recovery (5 % CO_2), determined using one-way repeated-measures ANOVA, indicated a significant difference between normocapnia and hypercapnia (***$P < 0.001$) and between hypercapnia and recovery (*$P < 0.05$). There was not a significant difference between firing frequencies during normocapnia and recovery periods ($P = 0.095$).

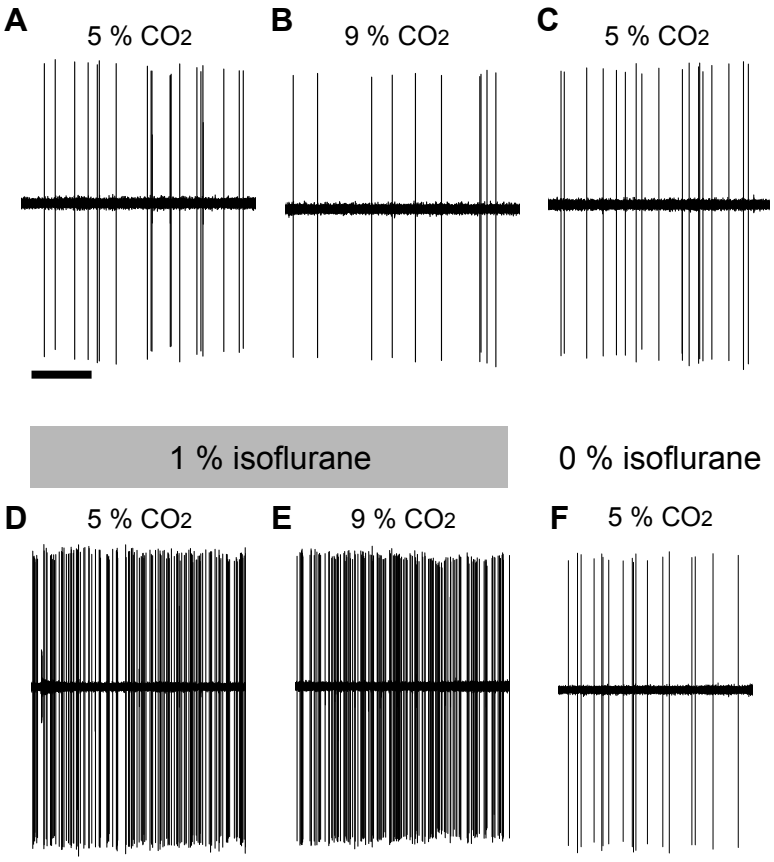

Figure 3.8. A CO_2-inhibited putative GABA neuron is no longer chemosensitive during 1 % isoflurane treatment. a) Extracellular recording of a medullary raphé neuron firing at 0.13 Hz at baseline (5 % CO_2; pH 7.4). Scale bar = 5 s. **b)** The neuron exhibited a 37.5 % decrease in firing frequency during hypercapnic challenge (9 % CO_2; pH 7.2). **c)** Firing frequency recovered to 0.15 Hz upon return to baseline conditions. **d)** The same neuron increased firing from 0.15 Hz to 2.58 Hz in response to 1 % isoflurane. **e)** During hypercapnic challenge, the neuron continued increasing its firing frequency to 2.68 Hz. **f)** The cell recovered toward baseline firing upon washout of isoflurane and return to normocapnia.

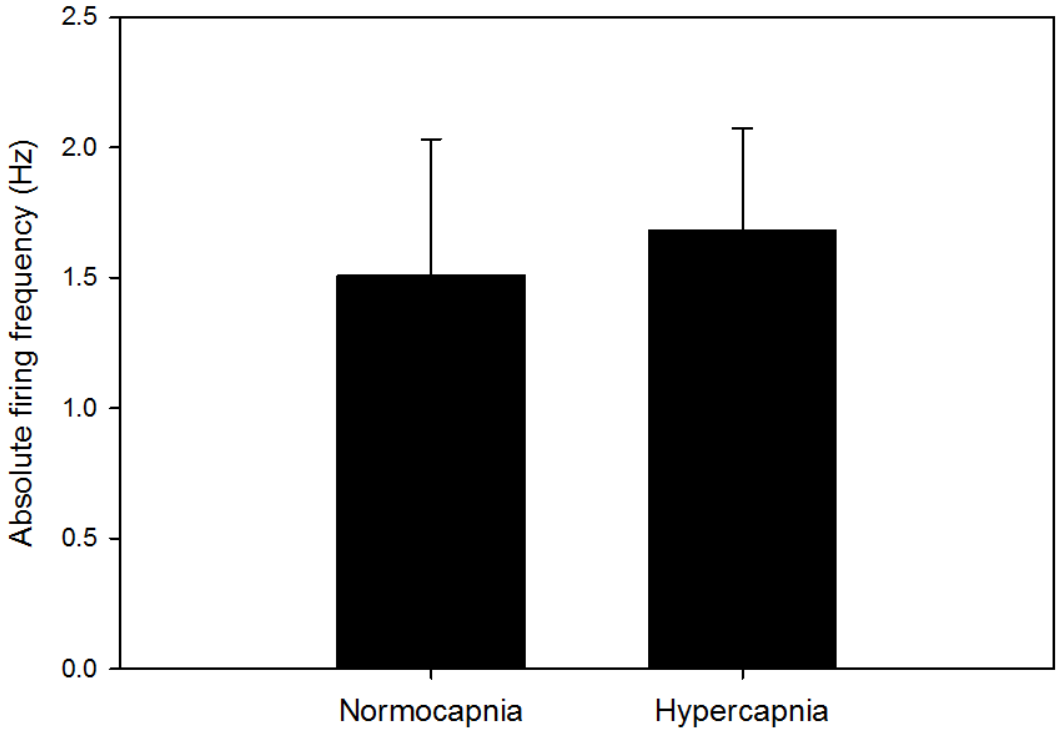

Figure 3.9. Isoflurane disrupts the chemosensitivity of CO_2-inhibited putative GABA neurons. Cells that increased firing frequency in response to isoflurane, which prior to isoflurane treatment were identified as CO_2-inhibited neurons, did not respond to the hypercapnic challenge during 1 % isoflurane treatment. During treatment with 1 % isoflurane, absolute firing frequency during normocapnia (5 % CO_2; pH 7.4) and hypercapnia (9 % CO_2; pH 7.2) were not significantly different, determined by paired *t*-test (n=4; $P = 0.510$).

Chapter 4

Conclusion

4.1 Summary of results

This graduate thesis presents research on the influence of the volatile anesthetic isoflurane on the action potential discharge and chemosensitivity of medullary raphé 5-HT and GABA neurons. Initially, this project aimed to address a long-standing controversy surrounding the identity of 5-HT neurons as central chemoreceptors, in which 5-HT neurons were shown to be stimulated by CO_2 *in vitro* (Richerson et al., 2001), *in situ* (Iceman et al., 2013), and *in vivo* studies on awake, freely-moving cats (Veasey et al., 1995; Veasey et al., 1997), but *in vivo* studies on rodents anesthetized with the halogenated volatile anesthetics, halothane and isoflurane, did not find 5-HT neurons to be chemosensitive (DePuy et al., 2011; Mulkey et al., 2004). We tested the hypothesis that isoflurane disrupts the chemosensitivity of medullary raphé 5-HT neurons *in situ*. In collaboration with *in vitro* and *in vivo* studies from the Richerson laboratory at the University of Iowa, we showed that isoflurane does, in fact, disrupt the chemosensitivity of 5-HT neurons (Massey et al., 2013). We suggest that these data offer a potential explanation for the contradictory findings surrounding the identity of 5-HT neurons as central chemoreceptors *in vivo*.

Following our study on the influence of isoflurane on the chemosensitivity of medullary raphé 5-HT neurons, we investigated how isoflurane affects action potential discharge of

5-HT neurons. We tested the hypothesis that isoflurane inhibits the baseline firing of medullary raphé 5-HT neurons *in situ*. We showed that isoflurane inhibited the baseline firing of caudal raphé 5-HT neurons in the descending 5-HT system. Isoflurane routinely inhibited action potential discharge in 5-HT neurons, or caused firing to cease completely. These results were consistent for all tested concentrations of isoflurane (1, 1.5, and 2 %), and the degree of inhibition increased as the concentration of isoflurane in extracellular solution increased. We found a significant difference between neuronal responses to 1 and 2 % isoflurane, which indicated dose responsiveness of 5-HT neurons to isoflurane within a clinically relevant range.

1 % isoflurane approximated 1.15 MAC, the minimum alveolar concentration for isoflurane necessary for surgical anesthesia (Eger, 1981). This concentration was confirmed by gas chromatography – mass spectrometry (GC – MS) analysis. At 10 min, the time point when all neuronal responses to isoflurane were recorded, the mean equilibrium isoflurane concentration was 0.38 mM, which was equivalent to 1 volume percent (0.38 mM = mean concentration from 6 to 10 min; 0.37 mM = 1 volume percent; Eilers et al., 1999). This GC – MS analysis confirmed that our results from experiments *in situ* are relevant to evaluating results from *in vivo* experiments on anesthetized animals, which typically are anesthetized within the range of 1 to 2 % inhaled anesthetic. Furthermore, confirming that the concentration of isoflurane received by the preparation was equivalent to 1.15 MAC allowed us to ensure that our results documenting the influence of isoflurane on 5-HT neurons are clinically relevant.

Having established the effect of isoflurane on the basal activity and chemosensitivity of 5-HT neurons, the proposed excitatory chemosensors in our "push – pull" model, we then focused on defining how isoflurane affects GABA neurons, which are the inhibitory chemosensors proposed in our model. We aimed to identify how isoflurane affects the basal activity and chemosensitivity of CO_2-inhibited putative GABA neurons in the medullary raphé. We tested the hypothesis that isoflurane enhances action potential discharge of medullary raphé CO_2-inhibited GABA neurons and disrupts their chemosensitivity *in situ*. We showed that CO_2-inhibited cells respond to isoflurane in one of two distinct ways: action potential discharge of these cells either dramatically increased, or significantly decreased, to the point of ceasing in the majority of cases, in response to 1 % isoflurane. Comparing the baseline firing characteristics of cells that were stimulated to cells that were inhibited by isoflurane, we found a significant difference in baseline firing rates, in which isoflurane-stimulated neurons fired slowly at baseline (mean firing frequency: 0.50 ± 0.38 Hz) compared to isoflurane-inhibited neurons (mean firing frequency: 2.72 ± 0.70 Hz). Our results point toward a heterogeneous population of medullary raphé CO_2-inhibited putative GABA neurons represented by at least two distinct groups, which differ in their response to isoflurane and baseline firing frequency: 1) "slow-firing" CO_2-inhibited putative GABA neurons that are stimulated by isoflurane; and 2) "fast-firing" CO_2-inhibited putative GABA neurons that are inhibited by isoflurane.

In terms of the influence of isoflurane on the chemosensitivity of CO_2-inhibited putative GABA neurons, we found that isoflurane disrupts chemosensitivity in the population of "slow-firing" isoflurane-stimulated CO_2-inhibited putative GABA neurons. Chemosensitive responses could not be resolved in the population of "fast-firing" isoflurane-inhibited CO_2-inhibited putative GABA neurons because these neurons ceased firing with isoflurane. These results indicating that isoflurane disrupts chemosensitivity in CO_2-inhibited isoflurane-stimulated putative GABA neurons are consistent with our previous findings that isoflurane disrupts chemosensitivity in medullary raphé CO_2-stimulated 5-HT neurons (Massey et al., 2013).

4.2 Relevance of this dissertation

Interpretation of results from in vivo *studies using anesthesia:* Collectively, these data on the influence of isoflurane on medullary raphé 5-HT and GABA neurons indicate that these two neuron types are profoundly affected by anesthetics. The fact that isoflurane caused significant changes in the action potential discharge of medullary raphé 5-HT and GABA neurons is imperative to consider when evaluating results from *in vivo* studies that use anesthesia during neuronal recording. For example, Mulkey et al. (2004) reported from *in vivo* recordings in rats maintained under 0.9 % halothane that 5-HT neurons fired within a range of 0.5 to 4.2 Hz at baseline (n=37; 27 cells were filled with biotinamide and 24 cells showed tryptophan hydroxylase immunocytochemical reactivity). The relatively fast maxima reported in the firing frequency range of 0.5 to 4.2 Hz (we observe serotonergic neurons firing within a range of approximately 0.5 to 3 Hz) may reflect a

subset of 5-HT neurons that are insensitive to anesthetics, perhaps due to a low expression of TASK channels. Alternatively, the relatively fast firing frequencies may reflect the fact that these 5-HT neurons were stimulated antidromically for selection of bulbospinal neurons, and spinally activating these neurons prior to recording may have caused them to have slightly higher baseline firing rates. As we have clearly demonstrated here, the halogenated anesthetic isoflurane, which has many properties similar to halothane, has a profound effect on 5-HT neurons as baseline. Thus, any analysis of the baseline firing frequency of the 5-HT neurons reported in Mulkey et al. (2004) is impeded by the fact that the animals were anesthetized with halothane throughout neuronal recording.

As a second example, DuPuy et al. (2011) reported the baseline firing rates of channelrhodopsin-2 (ChR2)-transfected serotonergic neurons from *in vivo* recordings in rats anesthetized with low concentrations (0.2 to 1 %) of isoflurane. Of the 19 cells, 11 were tonically active with baseline firing rates ranging from 0.1 to 4.4 Hz. Similar to Mulkey et al. (2004), their reported maxima is relatively high, which may either reflect a population of 5-HT neurons that are insensitive to isoflurane, or it may be due to the fact that these ChR2-transfected 5-HT neurons were identified by photostimulation prior to recording, which may have stimulated the firing rates observed at baseline. Importantly, of the 19 recorded cells, 8 were silent prior to photostimulation-induced depolarization. This population of 5-HT neurons may highly express TASK channels and may be

representative of the serotonergic neurons that are silenced by isoflurane, as characterized in this study.

Given that we observe marked inhibition of 5-HT neurons in response to isoflurane, it is plausible that *in vivo* experiments on anesthetized rats unknowingly target only those 5-HT neurons that are marginally sensitive or insensitive to isoflurane. These populations may express low levels of TASK channels, or isoflurane-insensitivity may be conferred by a different unknown mechanism. It is also plausible that some recordings misrepresent normal neuronal activity at baseline due to the artifact introduced by anesthesia. Our interpretation of the results from these and other *in vivo* studies must be viewed in light of the fact that we have demonstrated that isoflurane profoundly affects the firing characteristics of 5-HT and GABA medullary raphé neurons.

Evidence of a presynaptic site of anesthetic action: Following the transition to the hypothesis that anesthetics act on specific protein targets, particularly ion channels, the majority of research on molecular mechanisms of anesthetic action has centered on investigating how anesthetics affect $GABA_A$ receptors. As the most abundant inhibitory neurotransmitter receptor in the brain, $GABA_A$ receptors are a popular target for research targeting the question of how anesthetics produce overall central nervous system depression. However, a growing number of researchers recognize the likely contribution of presynaptic inhibition of neurotransmitter release to central nervous system depression during anesthesia (Griffiths and Norman, 1993; Pocock and Richards, 1993). Thus, a

growing body of literature describes anesthetic action on presynaptic sites that are involved in neurotransmitter release, including voltage-gated Na^+ channels (Herold and Hemmings, 2012), two-pore-domain background K^+ channels (Bayliss and Barrett, 2008), and voltage-gated Ca^{2+} channels (Orestes and Todorovic, 2010).

Anesthetics have been shown to inhibit neurotransmitter release through inhibition of voltage-gated Ca^{2+} currents (Study, 1994; Nikonorov et al., 1998; Kamatchi et al., 1999). Studies have also shown that anesthetics can inhibit neurotransmitter release through direct interaction with neurotransmitter release machinery (Herring et al., 2009), which is thought to involve anesthetics binding to vesicle fusion proteins, SNARE and SNARE-associated proteins (Xie et al., 2013). Perhaps most germane to the present study is evidence suggesting that anesthetics depress neurotransmitter release by inhibiting action potential conductance. Convincing evidence suggests that anesthetics inhibit action potential conductance through blockade of Na^+ channels (Ouyang and Hemmings, 2005; Wu et al., 2004). Additionally, anesthetics have been shown to inhibit action potential conductance by hyperpolarizing neurons through potentiation of TASK channel-mediated background K^+ currents (Patel et al., 1999; Sirois et al., 2000). Inhibition of action potential conductance through blockade of Na^+ channels and/or potentiation of background K^+ currents would lead to decreased action potential dependent neurotransmitter release, which could contribute to overall central nervous system depression during general anesthesia.

In the present study, we monitored action potential discharge before and during treatment with isoflurane. Observing how action potential discharge changes in response to isoflurane treatment allowed us to directly observe a presynaptic influence of anesthetics. Importantly, our evidence of a presynaptic influence of anesthetics on action potential discharge is demonstrated in a partially intact network, whereas the vast majority of prior studies on presynaptic anesthetic action are conducted in reduced *in vitro* or isolated synaptic bouton preparations. Our observation that isoflurane inhibits action potential discharge in medullary raphé 5-HT neurons may be relevant to mechanisms underlying overall central nervous system depression during anesthesia because decreased release of excitatory neurotransmitters, such as serotonin, would lead to depressed excitatory neurotransmission. Our observation that isoflurane stimulates action potential discharge in a subset of medullary raphé CO_2- inhibited putative GABA neurons may also lend insight into mechanisms describing central nervous system depression during anesthesia, as increased neurotransmitter release of inhibitory neurotransmitters, such as GABA, would lead to increased inhibitory neurotransmission. This work contributes to the growing body of literature that suggests that anesthetics affect action potential conductance and, thus, exert a presynaptic influence on neurotransmission.

Central mechanisms contributing to respiratory depression during anesthesia:

Respiratory depression is a well-known secondary effect of general anesthesia (Evers et al., 2006). All potent halogenated anesthetics, such as halothane and isoflurane, depress ventilation by reducing tidal volume, which is not compensated for by the concomitant

increase in respiratory rate, such that $PaCO_2$ increases (Torri, 2010). Anesthetics inhibit the ventilatory response to this increase in CO_2, but the mechanisms that give rise to the blunted ventilatory hypercapnic response are not completely defined. Convincing evidence suggests that low concentrations of anesthetics selectively affect peripheral chemoreceptors (Knill and Gelb, 1978; Knill and Clement, 1985; Dahan et al., 1994; van den Elsen et al., 1995), but the effect of volatile anesthetics on central chemoreceptors remains a pertinent question.

The Phox2b-expressing retrotrapezoid nucleus (RTN) neurons that have been well characterized as central chemoreceptors are known to be stimulated by isoflurane (Lazarenko et al., 2010). Enhanced action potential discharge in RTN neurons during isoflurane treatment is thought to contribute to maintenance of respiration during anesthesia since, although the response is blunted, there is some ventilatory response to hypercapnia during anesthesia. Previously, ours was the first study to document that isoflurane disrupted chemosensitivity in 5-HT neurons, the proposed central chemoreceptors in the medullary raphé (Massey et al., 2013). In the present study, we show that isoflurane disrupted chemosensitivity in the second population of cells that we identify as central chemosensors in the medullary raphé, the CO_2-inhibited putative GABA neurons. The fact that isoflurane disrupted the chemosensitivity of both phenotypes of medullary raphé central chemosensors, CO_2-stimulated 5-HT and CO_2-inhibited GABA neurons, may suggest a potential mechanism that contributes to the depressed ventilatory hypercapnic response during anesthesia; however, the fact that

there is some residual ventilatory response during anesthesia suggests that the central chemosensors proposed in our "push – pull" model of central chemosensitivity are not the only chemosensors that contribute to central chemosensitivity. Likely, RTN neurons stimulated by halogenated anesthetics, and perhaps other central chemoreceptors, play a larger role in maintaining the ventiliatory response to increased CO_2 during anesthesia. Our results suggest that the medullary raphé 5-HT and GABA neurons that we study may contribute to the overall blunted ventilatory hypercapnic response that occurs during anesthesia.

4.3 Future directions

Design of in vivo *experiments using anesthesia:* System CO_2 sensitivity is dampened under some types of anesthesia, particularly when using halogenated volatile anesthetics such as halothane and isoflurane. We studied CO_2-stimulated 5-HT and CO_2-inhibited GABA neurons and showed that chemosensitivity of these neurons is disrupted by isoflurane, which may contribute to blunted systemic CO_2 sensitivity during anesthesia. The fact that halogenated anesthetics significantly affect the chemosensitivity of raphé neurons, which may contribute to the blunted systemic ventilatory hypercapnic response, makes it difficult to interpret results from *in vivo* studies on central chemosensitivity that use anesthesia during neuronal recording. Although there are significant benefits to using anesthesia to maintain preparations for electrophysiological recording *in vivo*, future experimental design should be carefully considered in light of our results that show that

in fact, the chemosensitivity and basal activity of raphé neurons are significantly changed in the presence of anesthesia.

In contrast to the volatile anesthetics halothane and isoflurane, urethane is known to have minimal effects on respiration and systemic chemosensitivity (Hughes et al., 1982; Pagliardini et al., 2012). In fact, a single low dose (typically 1.0 to 1.5 g/kg) produces immobility for several hours while preserving normal circulation, respiration, and reflex responses (Koblin, 2002). Due to its minimal interference with homeostatic conditions, urethane is popular for use in animal experiments because data collected in animals maintained under urethane anesthesia is considered physiologically relevant. Although urethane is not administered to patients due to its mutagenic, carcinogenic, and hepatotoxic properties, and thus, studies on this agent are less clinically relevant, it would be useful to investigate the effect of urethane on 5-HT and GABA raphé populations for the purpose of designing future experiments aimed at investigating central chemosensitivity *in vivo*. Choice of concentration would be a key point in any potential future studies involving urethane, as larger doses (≥1.5 g/kg) depress respiratory function (Koblin, 2002). If low doses of urethane did not affect neuronal chemosensitivity, then those data would support use of urethane-anesthetized preparations in studying central chemosensitivity *in vivo*.

Investigating molecular mechanisms of anesthetic action: This research indicates that isoflurane significantly affects baseline activity of both 5-HT and GABA raphé neurons,

but the molecular mechanisms underlying these effects remain unknown. The potential mechanisms of anesthetic action proposed in this thesis should be explored. We proposed that inhibition of action potential discharge in 5-HT neurons in response to isoflurane may be due to isoflurane binding to TASK-1 and TASK-3 channels on 5-HT neurons. Potentiation of background potassium current through TASK channels would cause hyperpolarization and decreased neuronal excitability. An antagonist specific to TASK-1 and TASK-3 channels should be applied while recording from 5-HT neurons during isoflurane administration to test this hypothesis.

We also proposed that enhancement of action potential discharge in a subset of CO_2-inhibited putative GABA neurons may be due to the inhibition of a background K^+ current through THIK-1 channels. THIK-1 channels are inhibited by inhalational anesthetics (Rajan et al., 2001), and inhibition of the outward K^+ current through these channels leads to membrane depolarization and increased neuronal excitability. Thus, we postulate that inhibition of THIK-1 channels by isoflurane may cause the enhanced action potential discharge observed in a subset of CO_2-inhibited putative GABA neurons during isoflurane treatment. Although pharmacological methods have not yet been developed to target THIK-1 channels, there are antibodies to target these channels. CO_2-inhibited putative GABA neurons that are stimulated by isoflurane could be labeled with biotinamide by the juxtacellular labeling method, and then immunohistochemical methods could assess the presence of THIK-1 channels in these neurons. Collectively, these experiments would help define some of the molecular mechanisms of anesthetic

action that give rise to the changes in baseline activity observed in medullary raphé neurons during isoflurane treatment.

4.4. Summary

In conclusion, the research presented here indicates that the anesthetic isoflurane has a significant influence on neuronal baseline activity, in terms of action potential discharge, and chemosensitivity, in terms of a response to hypercapnic challenge, of medullary raphé 5-HT and GABA neurons *in situ*. We showed that isoflurane inhibited action potential discharge in medullary raphé 5-HT neurons, which added to our previous study indicating that isoflurane disrupts the chemosensitivity of these neurons (Massey et al., submitted, see appendix). We also showed that isoflurane stimulates action potential discharge in a subset of medullary raphé CO_2-inhibited putative GABA neurons and inhibits action potential discharge in a different subset of medullary raphé CO_2-inhibited putative GABA neurons. In the subset of CO_2-inhibited isoflurane-stimulated cells, isoflurane disrupted chemosensitivity of these putative GABA neurons.

This research will contribute to our understanding of how anesthetics affect action potential discharge in medullary raphé neurons, which may offer insight into mechanisms of general anesthesia. Our results indicating that medullary raphé neurons are profoundly affected by anesthetics, both in terms of their baseline firing and chemosensitive properties, will aid in interpretation of results from *in vivo* studies on anesthetized rodents and may inform future design of experiments that require anesthesia for conducting

neuronal recordings. Furthermore, by documenting how action potential discharge in medullary raphé 5-HT and GABA neurons is affected by isoflurane, we contribute to the growing body of literature describing presynaptic mechanisms of anesthetic action Finally, documenting how anesthetics affect the chemosensitivity of medullary raphé neurons that are known to be involved in respiratory circuits may help define molecular mechanisms underlying the decreased ventilatory response to hypercapnia that is characteristic of general anesthesia.

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Appendix A

Complete Methods

Experimental animals and preparations: Animal care and experimental procedures followed guidelines set by the National Institutes of Health Office of Laboratory Animal Welfare and the United States Department of Agriculture Animal Welfare Act. All protocols were in accordance with the University of Alaska Fairbanks Institutional Animal Care and Use Committee guidelines. Juvenile male rats (50 – 150 g; Sprague-Dawley strain; Simonson Laboratories) were used to generate the perfused *in situ* brainstem preparation as previously described (Paton, 1996; Corcoran et al., 2013). Animals were placed in an enclosed chamber, anesthetized with vaporized isoflurane (5 % isoflurane – 95 % O_2; Isoflurane Vapor 19.1; Butler Schein), and administered an intraperitoneal heparin sodium injection (0.5 mL of 1000 iu/ml ip; Baxter, Deerfield, IL) to prevent formation of blood clots during surgery. Following a 15-min recovery period, deep anesthesia was induced with 5 % isoflurane and assessed by the cessation of spontaneous breathing and absence of a withdrawal response to firm toe pinch and "retinal tap." Anesthesia was discontinued as preparations were bisected sub-diaphragmatically, decerebrated rostral to the superior colliculi, and skinned. The head and thorax were immersed in ice-chilled perfusate for the remainder of the dissection. The descending aorta was isolated, and the posterior throax and spinal cord were removed to mid-thoracic level to expose the heart.

Each preparation was placed in the recording chamber in a prone position. The descending aorta was cannulated with a double-lumen catheter, and preparations were retrogradely perfused with a solution at a temperature of 31 °C. The perfusate solution contained the following (in mM): 1.0 $MgSO_4$, 125 NaH_2PO_4, 4.0 KCl, 24 $NaHCO_3$, 115 NaCl, 10 D-Glucose, 2.0 $CaCl_2$, and 0.18 Ficoll. Baseline conditions, when the perfusate was equilibrated with 95 % O_2 – 5 % CO_2 (PCO_2 33 mmHg; pH=7.4), approximated normocapnic plasma *in vivo*. Levels of O_2 and CO_2 in the perfusate were controlled by equilibrating a perfusate reservoir with gas mixtures produced by a precision gas mixer (CWE GSM-3) and verified by a CO_2 analyzer (Applied Electrochemistry CD-3A). The perfusate passed through a heat exchanger to maintain temperature at 31 °C (Lauda E100 Water Bath), bubble trap to remove gas bubbles and minimize pulsations from the roller pump, and mesh filter to prevent debris from blocking capillary beds. The first 50 mL of perfusate that passed through the preparation was discarded to eliminate blood, and the remaining 450 mL of perfusate was recirculated. Following cannulation, vasopressin (200 μm; 5 μM Arg-vasopressin acetate salt in H_2O) was administered through the perfusate at approximately 3 min post-cannulation to stimulate an initial increase in pressure. Perfusion pressure, measured with a blood pressure transducer on the second lumen of the catheter, was gradually increased to 40-60 mmHg by regulating the speed of a peristaltic perfusion pump (Watson-Marlow 505Du). The pericardium was removed and the heart was tied off to prevent interference from heart pulsations during neuronal recordings. The preparation was tightly secured in a stereotaxic head frame using ear bars, nose bar, and mouthpiece, and once the preparation exhibited spontaneous

breathing, the neuromuscular blocker gallamine triethidodide (3 mL; 60 mg/L, Sigma-Aldrich) was added to the perfusate to eliminate movement. The occipital bone, surrounding musculature, and cerebellum were removed to expose the dorsal surface of the brainstem.

Extracellular recording and experimental treatments: Extracellular recordings of medullary raphé (r. magnus, r. obscurus, r. pallidus) neurons were made using single pulled glass capillary electrodes (1.2 mM; 15–30 MΩ; David Kopf Instruments 700 D) filled with biotinamide hydrobromide (5 % in 0.5 M sodium acetate, Life Technologies). The electrode was secured to the electrode-holder (caudally inclined 1.25 mm; David Kopf Instruments), positioned near the brainstem midline (≤ 0.1 mm lateral) 0 to 3 mm rostral of obex, and advanced through the brain tissue in 2 μm increments using a fine stepping motor (Burleigh Instruments Inchworm). Extracellular recordings were made with an intracellular amplifier (Axon Instruments Multiclamp 700B) in current clamp mode, with a high pass filter at 300 Hz and low pass filter at 1 kHz bessel with an high impedance headstage (Axon CV7B, Molecular Devices). Action potentials were digitized using Spike 2 (Cambridge Electronic Design Power 1401) and LabChart (AD Instruments) software and stored as computer data files.

Neuronal recordings were initiated under baseline conditions (95 % O_2 – 5 % CO_2; PCO_2 33 mmHg; pH=7.4). As stated above, these conditions approximate normocapnic plasma *in vivo*; however, due to the lack of hemoglobin, the solution hyperoxia (PO_2 ~ 600

mmHg) was necessary to maintain O_2 content sufficient to meet tissue metabolic demands, and this unavoidable hyperoxia was constant under all conditions. The normocapnic condition was followed by a hypercapnic challenge (91 % O_2 – 9 % CO_2; PCO_2 60 mmHg; pH 7.2; 5 min) to approximate conditions similar to those in plasma during a 4 % increase in inspired CO_2. The preparation was then returned to normocapnic conditions for an additional 5 min. The 5-min exposure was demonstrated to be sufficient for brain tissue equilibration with the 91 % O_2 – 9 % CO_2 gas levels in the perfusate (Wilson et al., 2001).

Spontaneously active neurons were identified electrophysiologically and classified according to changes in firing frequency elicited by elevation of arterial CO_2 concentrations during hypercapnic challenges (increase, decrease, and no change in baseline firing frequency). Following a return to baseline conditions for an additional 5 min, both chemosensitive and insensitive neurons were administered isoflurane. Isoflurane was bubbled into the perfusate for 10 min (1, 1.5, or 2 % isoflurane in 95 % O_2 – 5 % CO_2; percent isoflurane refers to volume percent). The volume percent was held constant by the isoflurane vaporizer (Isoflurane Vapor 19.1, Butler Schein). For chemosensitive neurons, the 5-min hypercapnic challenge was repeated in the presence of isoflurane (1, 1.5, or 2 % isoflurane; 91 % O_2 – 9 % CO_2). For insensitive neurons, hypercapnic challenges were not repeated. Upon return to baseline conditions (0 % isoflurane; 95 % O_2 – 5 % CO_2), the neuron was allowed to recover toward its baseline

firing frequency. Recovery times ranged from 10-30 min, depending on the individual neuronal characteristics and assays performed.

Juxtacellular labeling: Subsets of recorded neurons were individually filled with biotinamide using the juxtacellular labeling method (Pinault, 1996; Winkler et al., 2006). Extracellular recordings were conducted in current clamp mode (Axon Multiclamp 700B) to allow current to be injected through the electrode while action potentials were monitored. Positive current pulses were applied through the electrode (400-ms duration) and gradually increased from 0-15 mV (0.5 mV steps; Grass Stimulator S44B) until the cell was entrained to fire simultaneously with the applied stimulus. Injected current, ejection of biotinamide, and cellular firing entrainment caused uptake of the biotinamide marker by the recorded cell. Entrainment was maintained for at least 30 s. After termination of entrainment, the biotinamide was allowed to disperse for 30 min. Rats were perfused through the descending aorta with fixative [4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer saline (PBS); pH 7.4; Sigma-Aldrich]. Brainstems were then removed and stored in fixative prior to sectioning. Coronal sections (60 μm) were cut through the medulla (Vibratome 1000Plus), and sections were processed free-floating.

Immunohistochemistry: Coronal tissue sections were blocked and permeabilized in blocking buffer [Blocking buffer: 5 % normal goat serum (NGS in 0.1 % sodium axide; Caltag Spec. No. L10000, Lot: 384935A) and 0.3 % Triton X-100 in 0.1 M PBS] for 1 hr at 22 °C. Sections were incubated with a streptavidin-Alexa 546 conjugate (Life

Technologies #S-11225; 1:500 dilution in PBS with 5 % NGS) for 2 hrs at 22 °C to reveal biotinamide introduced into single neurons by juxtacellular labeling. To identify serotonergic cells, we used an antibody for the 5-HT-synthesizing enzyme tryptophan hydroxylase (TpOH). Sections were incubated overnight at 4 °C with mouse monoclonal anti-TpOH (Sigma-Aldrich #T0678; 1:1000 dilution in blocking buffer), followed by incubation for 1 hr at 22 °C. To identify GABAergic cells, we used an antibody for the GABA-synthesizing enzyme glutamate decarboxylase (GAD-67). Sections were incubated for 48 hrs at 22 °C in mouse monoclonal anti-GAD67 (Millipore MAB5406; 1:500 dilution in blocking buffer). Sections were incubated for 2 hrs at 22 °C in a secondary goat anti-mouse Alexa 488 antibody (Life Technologies #A-11029; 1:500 dilution in 0.1 M PBS with 5 % NGS) to label TpOH-immunoreactive and GAD67-immunoreactive cells. Sections were air-dried for 30 min, mounted with Vectashield (Vector Labs), and coverslipped. Low-magnification (10x) images were used to determine the location of biotinamide-labeled cells in relation to anatomical landmarks. Local biotinamide, 5-HT, and GABA fluorescence identified colocalization of serotonergic or GABAergic markers in biotinamide-labeled neurons. Fluorophores were individually excited and emission spectra were collected separately to minimize interference using a Zeiss LSM510 confocal microscope: biotin-labeled neuron, Alexa 546, 543 nm laser, filter BP 560-615; anti-TpOH, Alexa 488, 488 nm laser, filter BP505-530; anti-GAD-67, Alexa 647, 633 nm laser, filter LP650. Images are presented as a 40x projection of a z-stack.

Gas chromatography – mass spectrometry: All gas chromatography – mass spectrometry (GC – MS) analyses were performed on an Agilent Technologies 7890A GC System and 5975C mass selective detector with Triple Axis Detector. Helium carrier gas was used at a constant flow rate of 20 mL min^{-1}. A sample of 250 µL was injected into a DB-1 ms capillary column (45 m x 0.25 mm, film thickness 0.25 µm) at a split ratio of 50:1. The system oven temperature was initially maintained at 30 °C for 6 min, and then increased at a rate of 10 °C min^{-1} from 30 to 120 °C. MS acquisition was performed in full scan mode in the 40 to 200 amu mass ranges. Mass conditions were as follows: ionization voltage, 69.9 eV; ion source temperature, 230 °C; detector voltage, 1.6 kV.

Isoflurane was continuously bubbled into the perfusate for the duration of isoflurane treatment at a volume percent held constant by the isoflurane vaporizer (Butler Schein; Isoflurane Vapor 19.1). Isoflurane is poorly soluble in water but highly soluble in plastic and rubber tubing (Eger, 1981), so samples of the perfusate were analyzed by GC – MS to confirm that the experimental preparation received the intended concentration of isoflurane through the perfusate. A saturated solution of isoflurane was prepared by adding aliquots of isoflurane to perfusate solution and stirring in an airtight container for 3 hr (15 mM; Scheller et al., 1997). Different concentrations were prepared by diluting the saturated solution in perfusate (1:2, 1:4, 1:8, 1:16, 1:32, and 1:64) and 250 µL of headspace gas from each dilution was injected directly onto the GC-MS. The mean peak area for each dilution, corresponding to the number of isoflurane molecules in solution,

was used to generate a standard curve for isoflurane. The standard curve was fit to a linear regression line (SigmaPlot).

Samples of perfusate were taken from the aortic cannula, to best approximate the sample received by experimental preparations, at each minute during a 10-min 1 % isoflurane exposure. Additional samples were taken at 11 and 15 min to confirm that isoflurane concentrations were stable throughout the period of recording. GC – MS analysis of these samples yielded a value for mean peak area, corresponding to the number of isoflurane molecules in solution. The concentration of isoflurane in solution at each minute of the drug exposure was calculated from the standard curve equation. These calculated concentrations were used to confirm that at 10 min, the time point when all neuronal responses to isoflurane were recorded, the concentration of isoflurane in the perfusate was equivalent to 1 volume percent.

Data analysis: We discriminated individual extracellular unit activity using computer spike sorting software (Spike 2, CED and LabChart, AD Instruments). Stable 1- to 2-min periods of single-unit firing were analyzed before (baseline), during (hypercapnia), and after (recovery) hypercapnic challenge to provide a mean value for unit firing frequency (spikes/s), mean interspike interval (ms), standard deviation and standard error of mean interspike interval, and spike width. Subset analysis was used to classify neurons as chemo-stimulated where relative frequencies increased by greater than 20 % during hypercapnia and returned toward baseline firing during recovery, chemo-inhibited where

relative frequencies decreased by greater than 20 % during hypercapnia and returned toward baseline firing during recovery, and chemo-insensitive where relative frequencies changed by less than 20 % (Wang et al., 2001). We have previously reported the influence of isoflurane on hypercapnic responses in chemo-stimulated 5-HT neurons (Johansen et al., 2012; Massey et al., submitted). The present data set includes only chemo-insensitive neurons.

Data analysis – CO_2-insensitive putative 5-HT neurons: Cells were initially screen for being serotonergic by a real-time assessment of firing frequency and regularity. Neurons with firing frequencies between approximately 0.5 and 3 Hz that fired regularly, without sustained pauses or bursts in firing, were regarded as putative 5-HT neurons during experimental recording. Putative 5-HT neurons were confirmed as such with the following algorithm, in which the mean interspike interval ($\overline{X}$, in ms) and standard deviation of the intervals (sd, in ms) are considered independent variables: $Y(\overline{X},sd) = 146 - \overline{X} + 0.98sd$ (Mason, 1997). If the value of this function is less than zero [$Y(\overline{X},sd) < 0$], then the cell was confirmed serotonergic, and if the value of the function is greater than zero [$Y(\overline{X},sd) > 0$], then the cell was considered nonserotonergic. All putative 5-HT neurons that were identified by real-time assessment of firing pattern and regularity for recording were confirmed serotonergic cells by the algorithm.

All putative 5-HT neurons (n=38) were administered a single dose of isoflurane [1 % (n=18); 1.5 % (n=10); and 2 % (n=10)]. For each dose, the mean firing frequency at

baseline (0 % isoflurane) and mean firing frequency in the last minute of isoflurane exposure (1, 1.5, or 2 % isoflurane) were compared using a paired *t*-test. Baseline firing frequencies for each of the three groups (1, 1.5, and 2 % isoflurane) were compared by one-way ANOVA to confirm that mean firing frequencies under baseline conditions were not significantly different from each other. To assess the existence of a dose response, percent change from baseline during isoflurane exposure was compared between isoflurane treatment groups using a one-way ANOVA. Post-hoc tests used Holm-Sidak pairwise comparison (SigmaPlot). Throughout the text, values are expressed as means ± standard error of the mean. The criteria for statistical significance was $P < 0.05$, and instances of $P < 0.01$ and $P < 0.001$ are also noted.

Data analysis – CO_2-inhibited putative GABA neurons: For cells identified as CO_2-inhibited based on a minimum 20 % decrease in firing frequency during hypercapnic challenge, between groups comparison of the change in absolute firing frequency from normocapnia (5 % CO_2) to hypercapnia (9 % CO_2) and recovery (5 % CO_2) was determined using one-way repeated-measures ANOVA. Post-hoc tests used Holm-Sidak pairwise comparison (SigmaPlot).

Considering all CO_2-inhibited neurons as a single group (n=19), between groups comparison of baseline firing frequency to firing frequency during 1 % isoflurane treatment was determined using a paired *t*-test. However, this analysis of the entire population of CO_2-inhibited putative GABA neurons did not reflect the occurrence of

neurons that increase firing in response to 1 % isoflurane. Neurons were subsequently analyzed by percent change from baseline firing during isoflurane, to reveal a bimodal distribution represented by two groups: 1) “slow firing” cells that increase firing during isoflurane and 2) “fast firing” cells that decrease or cease firing during isoflurane. Between groups comparison of the mean percent change from baseline of "slow firing" and "fast firing" CO_2-inhibited neurons was determined by *t*-test. Between groups comparison of mean baseline firing frequencies of "slow firing" and "fast firing" CO_2-inhibited neurons was determined by one-tailed *t*-test. The one-tailed *t*-test was used because the distribution of firing frequencies was truncated at zero, corresponding to a neuron that is not firing (0 Hz), and therefore, the distribution was not normal and a two-tailed *t*-test was not appropriate for analysis. The between groups comparison of mean baseline firing frequencies was also determined by a *t*-test on ranks by the same justification of a distribution that is truncated at zero. Throughout the text, values are expressed as means ± standard error of the mean. The criteria for statistical significance was $P < 0.05$, and instances of $P < 0.01$ and $P < 0.001$ are also noted.

Appendix B

Co-authored manuscript: Isoflurane abolishes the response of serotonin neurons to CO_2/pH

The full submitted manuscript is attached on the following pages

Isoflurane abolishes the response of serotonin neurons to CO_2/pH

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Running Title: Isoflurane blocks 5-HT neuron chemosensitivity

Key Words: *TASK, Patch clamp, Breathing, Chemosensitivity, Raphe*

Central CO_2 chemoreceptors are critically important elements of the respiratory control system, but the identity of these cells is unknown and highly controversial. Serotonin (5-HT) neurons have properties expected of chemoreceptors, except that they are insensitive to CO_2/pH *in vivo* in animals anesthetized with halothane or isoflurane. Here we found that isoflurane markedly impaired the hypercapnic ventilatory response *in vivo*. 5-HT neurons in culture and in an *in situ* perfused brainstem were stimulated by hypercapnia. Isoflurane completely abolished 5-HT neuronal chemosensitivity in both preparations, and hyperpolarized 5-HT neurons consistent with activation of leak K^+ channels. However, these channels were not involved in the underlying mechanism of chemosensitivity. These data resolve a major controversy about respiratory chemoreception, and provide missing evidence supporting the hypothesis that 5-HT neurons are central chemoreceptors. We conclude that halogenated anesthetics can introduce a serious artifact into *in vivo* studies of control of breathing.

INTRODUCTION

Breathing is controlled by a network of neurons within the brainstem that generate rhythmic motor output to respiratory muscles. One of the most important sources of feedback for regulation of tidal volume (V_T) and respiratory frequency (F_R) comes from central respiratory chemoreceptors (CRCs) that monitor arterial PCO_2 indirectly via brain tissue pH[1-3]. The location and identity of the CRCs has been the subject of considerable controversy, with multiple sites proposed to contain putative pH/CO_2 sensors[1-6], but there is significant disagreement about whether all of these neurons are chemoreceptors or the relative importance of each.

There is strong evidence from *in vivo* and *in vitro* approaches that serotonin (5-HT) neurons in the medulla are CRCs[1-4, 6-22]. The hypothesis that 5-HT neurons are CRCs has generated considerable support, and would be widely adopted except for the observation that hypercapnia does not consistently stimulate 5-HT neurons *in vivo*[5, 23]. For example, recordings from the raphé obscurus[18] and dorsal raphé[19] in unanesthetized, behaving cats demonstrate that a subset of neurons presumed to be serotonergic increase their firing frequency in response to as little as 3% inhaled CO_2. In contrast, extracellular recordings from the raphé obscurus and ventrolateral medulla (VLM) of anesthetized rats *in vivo* indicate that 5-HT neurons do not increase their firing frequency in response to inhalation of up to 10% CO_2[5, 23]. It is important to determine why such different results were obtained in two relatively similar preparations. Single unit recordings *in vivo* are the gold standard for defining normal neuronal activity, and these four studies[5, 18, 19, 23] are the only ones to use this

approach to study chemosensitivity of 5-HT neurons. A potential confounding variable is that in the two studies failing to show 5-HT neuron chemosensitivity, animals were anesthetized with halothane or isoflurane[5, 23]. These anesthetics potentiate TASK channel currents[24] and these channels are highly expressed on 5-HT neurons[25]. TASK channels mediate a leak K^+ current, which causes hyperpolarization of membrane potential (E_m). Thus, these anesthetics would inhibit respiratory output at multiple sites, including 5-HT neurons in particular. Consistent with this, the hypercapnic ventilatory response (HCVR) reported by DePuy *et al.*[23] in isoflurane anesthetized rats was limited to an increase in ventilation ($\dot{V}_E$) of only 35% with inhalation of 10% CO_2. In contrast, it is characteristic for $\dot{V}_E$ to increase by more than 250% in unanesthetized mice and rats exposed to only 7% CO_2[10, 17, 26]. This reduced responsiveness to CO_2 is disconcerting and suggests a defect in the HCVR in the two studies that used halogenated anesthetics[5, 23]. Resolving this controversy is critical to evaluate the veracity of the hypothesis that 5-HT neurons are CRCs.

Here we found that isoflurane strongly depressed the HCVR *in vivo*. We also found that isoflurane profoundly inhibited firing of 5-HT neurons in cell culture and in an isolated perfused brainstem preparation, and abolished chemosensitivity of 5-HT neurons in both preparations. These data demonstrate that halogenated anesthetics introduce a severe artifact in studies of respiratory chemoreception or 5-HT neuron chemosensitivity. Accurate conclusions cannot be made about CRCs or neural control of breathing on the basis of experiments using these anesthetics.

RESULTS

Isoflurane severely impaired the hypercapnic ventilatory response *in vivo*

To quantify the effect of isoflurane on chemoreception, we measured the HCVR in awake (unanesthetized) animals (Fig. 1a) and again following at least 15 minutes of exposure to 1% isoflurane (Fig. 1b). Increases in inspired CO_2 from 0% to 3%, 5% and 7% were each performed with 50% O_2 (balance N_2) to reduce the contribution from peripheral chemoreceptors[10]. $\dot{V}_E$ increased by 338% in unanesthetized mice in response to 7% CO_2 (from 2.52 ± 0.86 µl g^{-1} min^{-1} to 11.03 ± 2.95 µl g^{-1} min^{-1}; $p<0.0001$; Fig. 1c). Isoflurane (1%), which is the approximate concentration needed to reach 1 MAC (mean alveolar concentration required for surgical anesthesia)[27], had no effect on baseline breathing ($p=0.63$). However, the anesthetic severely blunted the HCVR. In response to 7% CO_2, $\dot{V}_E$ increased by only 83% (from 2.23 ± .61 µl g^{-1} min^{-1} to 4.08 ± 1.31 µl g^{-1} min^{-1}; a 75% reduction in the HCVR with isoflurane, Fig. 1c); $\dot{V}E$ in 7% CO_2 was significantly reduced in the presence of isoflurane ($p<0.0001$) as compared to 7% CO_2 in the absence of isoflurane. Isoflurane had a particularly profound effect on the F_R component of the HCVR (Fig. 1d). In awake mice, when CO_2 was increased to 7%, F_R increased by 85% (from 150.85 ± 22.67 breaths min^{-1} to 279.71 ± 38.95 breaths min^{-1}; $p<0.0001$). In 1% isoflurane, F_R no longer changed with hypercapnia ($p=0.92$). Isoflurane also blunted the effect of CO_2 on V_T, albeit less than on F_R (Fig. 1e). In awake mice, V_T increased 139% (from 16.43 ± 3.80 µl g^{-1} to 39.32 ± 7.67 µl g^{-1}) while in 1% isoflurane, V_T only increased 89% (14.75 ± 3.19 µl g^{-1} to 27.85 ± 8.40 µl g^{-1}). V_T in

7% CO_2 was significantly reduced in the presence of isoflurane as compared to 7% CO_2 in the absence of isoflurane ($p<0.0001$).

Isoflurane inhibited firing of 5-HT neurons *in vitro* and abolished their response to CO_2

To examine the effect of isoflurane on 5-HT neuron chemosensitivity, we performed perforated patch recordings from medullary raphé 5-HT neurons cultured from ePet-EYFP mice[28] (Fig. 2a). For the 5-HT neuron shown in Figure 2b, acidic artificial cerebrospinal fluid (aCSF) (pH 7.15) dramatically increased firing frequency. This effect quickly reversed with a return to aCSF (pH 7.4). Isoflurane (1%) in aCSF eliminated 5-HT neuron firing, and acidosis no longer caused increase in firing. Firing was restored in aCSF upon washout of isoflurane and acidosis again caused an increase in firing frequency.

The effect of isoflurane was highly consistent across all recorded neurons (n=15; Fig. 2c). In the absence of isoflurane, acidosis consistently increased firing frequency in all 5-HT neurons tested (from 0.43 ± 0.25 Hz in aCSF to 1.90 ± 1.30 Hz in acidic aCSF, mean increase of 342%; $p<0.0001$; n=15), consistent with the degree of 5-HT neuron chemosensitivity previously reported[6, 8, 20-22]. When neurons were exposed to 1% isoflurane in aCSF, the firing frequency decreased from 0.43 ± 0.25 Hz to 0.00 ± 0.01 Hz (n=15). Furthermore, isoflurane abolished chemosensitivity of 5-HT neurons; in isoflurane, there was no change in firing rate in response to acidosis (from 0.00 ± 0.01 Hz to 0.12 ± 0.22 Hz; $p=0.66$; n=15) (Fig. 2c). The

inhibition of 5-HT neurons was reversible upon washout of isoflurane (n=12).

Isoflurane did not affect the underlying mechanism of chemosensitivity in 5-HT neurons

This protocol (Fig. 2b) did not distinguish whether isoflurane interfered with an underlying chemosensory mechanism, or hyperpolarized 5-HT neurons and prevented acidosis from reaching threshold for action potential generation. To differentiate between these possibilities, we examined changes in membrane potential (E_m) in response to isoflurane. In aCSF, isoflurane (1%) hyperpolarized 5-HT neurons by -6.52 ± 3.94 mV (Fig. 2d, left bar; n=15) relative to aCSF without isoflurane. Isoflurane in acidic aCSF hyperpolarized E_m by -2.45 ± 4.07 mV (Fig. 2d, right bar n=15) relative to aCSF without isoflurane. Differences in these E_m values indicate that acidosis could not overcome the hyperpolarization induced by isoflurane. Thus, 5-HT neurons retain sensitivity to acidosis, but isoflurane prevents their responsiveness.

Chemosensitivity of 5-HT neurons was then studied after the hyperpolarization induced by isoflurane was reversed by inducing an equal and opposite depolarization with current injection. 5-HT neurons were first exposed to acidic aCSF to quantify their chemosensitivity (Fig. 2e). Bath solution was then switched to aCSF with 1% isoflurane which hyperpolarized neurons and ceased their firing. Sufficient current was then injected to return firing frequency back to baseline.. When so treated, despite continued exposure to isoflurane neurons

increased firing when exposed to acidic aCSF, and did so to a degree equal to that in the absence of isoflurane (Fig. 2f). Thus, when hyperpolarization induced by isoflurane was overcome by current injection, the underlying chemosensitivity was neither exaggerated nor blunted, suggesting that chemosensory mechanisms were unaltered.

5-HT neurons were chemosensitive in a perfused brainstem preparation

It has been suggested that chemosensitivity of 5-HT neurons is an artifact of culture, and that it does not occur to a significant degree in an intact nervous system[23]. The abnormal cytoarchitecture in cell culture has been proposed to result in a decrease in glial buffering[23], causing neurons to be exposed to larger changes in pH during hypercapnia. In contrast, it has been proposed that the normal glial microenvironment in the intact brain would prevent large changes in pH in most regions[29] (including the raphé), while allowing larger changes in pH in a few specialized regions (such as the retrotrapezoid nucleus; RTN) where uniquely important chemoreceptors are located. To address the possibility that 5-HT neurons lack a robust chemoresponse when the glial microenvironment is intact, we conducted experiments in an unanesthetized acutely perfused *in situ* rat brainstem preparation. This preparation maintains an intact and functional respiratory control network, a normal glial microenvironment and cardiorespiratory reflexes similar to those *in vivo*[30, 31]. To determine whether 5-HT neurons are chemosensitive in an intact respiratory network, recordings were made from 5-HT neurons *in situ* (Fig.

3a). These neurons were identified as presumably serotonergic using electrophysiological criteria described by Veasey *et al.*[18, 19, 32]. Mason[32] and Mulkey *et al.*[5] confirmed the validity of these criteria by labeling recorded neurons, and demonstrated that electrophysiological characteristics correctly identify 5-HT neurons with approximately 90% accuracy. When the preparation was supplied with hypercapnic perfusate (pH 7.2) (Fig. 3b), the 5-HT neuron increased its firing frequency by 33% (from 0.9 Hz to 1.2 Hz) compared to control perfusate (pH 7.4). This effect reversed when the perfusate was switched back to control (Fig. 3c). An overlay of triggered spikes verified that this was a single unit recording (Fig. 3d). Juxtacellular labeling and subsequent immunohistochemistry demonstrated that the recorded neuron was tryptophan hydroxylase (TpOH) immunoreactive (Fig. 3e). These results refute the argument that chemosensitivity of 5-HT neurons is an artifact of culture. 5-HT neurons are chemosensitive in this *in situ* preparation in which the glial microenvironment is intact. These results agree with those of Veasey *et al.*[18, 19] who demonstrated chemosensitivity of 5-HT neurons in unanesthetized cats *in vivo*, and our own observations of 5-HT neuron chemosensitivity in acute rat and mouse brain slices[6, 8, 21]. These results provide the major piece of support that had been missing for the 5-HT neuron chemoreceptor hypothesis – documentation in an intact mammalian nervous system of chemosensitivity in neurons verified to be serotonergic by anatomical methods.

Isoflurane abolished chemosensitivity of 5-HT neurons in the perfused brainstem

We next determined whether isoflurane affected firing frequency of 5-HT neurons in a perfused brainstem. In control perfusate (pH 7.4), isoflurane (1%) reduced (Fig. 4a) or completely eliminated (Fig. 4b-c) spontaneous firing in 64% (n=14/22) or 36% (n=8/22) of 5-HT neurons, respectively. This effect was reversible (Fig. 4a and d). Illustrated neurons were verified to be serotonergic using juxtacellular labeling followed by TpOH immunohistochemistry (Fig. 4e and 4f).

Isoflurane (1%) also eliminated chemosensitivity of 5-HT neurons in the *in situ* perfused brainstem preparation. As with our previously described experiment, extracellular recordings from neurons (n=9) meeting the electrophysiological criteria for 5-HT neurons in the rostral medullary raphé (r. magnus and r. pallidus) revealed that these cells increased their firing frequency when control perfusate was changed to hypercapnic perfusate (Fig. 5 a-c), confirming that 5-HT neurons were chemosensitive *in situ*. The response was sizable (71% ± 56% increase from control), and consistent with the sensitivity of 5-HT neurons documented in cats *in vivo* [18, 19] and the response of the respiratory system as a whole in this preparation[31]. As above, exposure to 1% isoflurane in control perfusate eliminated (n=3/9) or markedly decreased (n=6/9) firing frequency and completely eliminated the response to hypercapnic perfusate (Fig. 5 d-e). This effect of isoflurane was reversible (n=8/9; Fig. 5c). Juxtacellular labeling and TpOH immunostaining confirmed that this was a 5-HT neuron (Fig. 5f). These results provide an

explanation for why 5-HT neurons have been found unresponsive to CO_2/pH during two of the previous four published studies that used single unit recordings *in vivo* to address this question[5, 23]. In both cases halogenated anesthetics were used, and such anesthetics abolished 5-HT neuron chemosensitivity.

DISCUSSION

5-HT neurons have properties consistent with CO_2 chemoreceptors

There are certain properties that are essential for neurons or glia to be CRCs[1]. These include that they are intrinsically chemosensitive to small physiologically relevant changes in CO_2[1, 3, 4, 6, 8, 15, 20, 22], and the change in firing frequency induced by hypercapnia would stimulate respiratory output[23].

There are other properties that 5-HT neurons have that make them well suited to be CRCs. 5-HT neurons are closely associated with the basilar artery and its largest branches[8], where they can accurately monitor arterial blood PCO_2. There is a dense concentration of 5-HT immunoreactive nerve terminals[33] and 5-HT receptor expression[34] in respiratory nuclei. In support of the hypothesis that 5-HT neurons are CRCs, focal acidosis in the raphé nuclei causes increased respiratory output *in vivo* and *in vitro* [2, 7, 9, 14, 35]. Disruption of 5-HT neurons by neurotoxins[13, 36] or nonselective agents such as muscimol[12] depresses the HCVR. Mice with genetic deletion[10, 11] or acute silencing[15] of 5-HT neurons have a blunted HCVR. The HCVR of rodents also develops with age in parallel with chemosensitivity of 5-HT neurons[21, 26].

Until recently it was unclear whether 5-HT neurons stimulate or inhibit breathing, only have a modulatory effect on respiratory output, or are required for plasticity[37, 38]. It has now been unequivocally demonstrated that 5-HT neurons stimulate the respiratory network at multiple sites. For example, 5-HT_{2A} receptor activation is required for generation of respiratory output from brain slices containing the pre-Bötzinger complex (PBC)[14, 39]. This is due in part to a requirement for 5-HT in expression of bursting pacemaker activity in a subset of neurons of the mouse PBC[14]. Thyrotropin-releasing hormone (TRH), which is co-localized in some 5-HT neurons, also induces bursting pacemaker activity in the dorsal respiratory group of guinea pigs[40]. Genetic deletion of 5-HT neurons leads to decreased respiratory output and periods of apnea in *Lmx1b*$^{f/f/p}$ neonates[11]. Exogenous application of 1-[2,3-dimethoxy-4-iodophenyl]-2- aminopropane (DOI), a 5-$HT_{2A/1C}$ agonist, increases $\dot{V}_E$ in neonatal *Lmx1b*$^{f/f/p}$ mice[11] and rats *in vivo* [41]. Selective stimulation of 5-HT neurons in the medullary raphé *in vivo* using optogenetics, increases phrenic nerve activity[23]. Thus, it is now well established that medullary 5-HT neurons stimulate respiratory output.

Hypercapnia stimulates 5-HT neurons *in vivo*

There exists a long-standing controversy regarding chemosensitivity of 5-HT neurons *in vivo*. Mulkey *et al.*[5] reported that 5-HT neurons in the VLM do not respond to inhalation of 10% CO_2 in halothane anesthetized rats *in vivo*. More recently DePuy *et al.*[23] found that 5-HT neurons in the raphé obscurus also do not

respond to 10% CO_2 in isoflurane anesthetized rats *in vivo*. From these data it was concluded that acid sensitivity of 5-HT neurons "is only notable in culture. It is inconsistent in slices, and these cells do not respond to hypercapnia *in vivo*."[23] The explanation for why 5-HT neurons are chemosensitive in culture and slices, but are unresponsive to hypercapnia *in vivo* was proposed to be due to stronger buffering of extracellular pH by glia in an intact brain, and this was said to be more effective in the raphé than other brain regions[23]. It is true that glial buffering can have a powerful effect on brain pH and within brain slices[29]. This could explain in part the larger responses of 5-HT neurons in culture than in slices, in situ or *in vivo*. However, hypercapnia *in vivo* actually induces a larger change in pH in the medullary raphé than in other regions of the brain[42], and pH responses in slices are smaller than in culture primarily because most recordings made in slices have been from young neurons (<P18) when chemosensitivity is not fully mature[21].

A greater degree of glial buffering also does not explain why Veasey *et al.*[18, 19] found that a subset of 5-HT neurons in both the medulla and midbrain of unanesthetized cats respond to inhalation of as little as 3% CO_2, and some nearly double their firing frequency in response to 8% CO_2. The experiments of Veasey *et al.* [18, 19] identified neurons as serotonergic using electrophysiological criteria instead of the juxtacellular labeling used by Mulkey *et al.*[5], but using even a subset of these electrophysiological criteria is approximately 90% accurate[5, 32]. Medullary raphé neurons have also repeatedly been shown to be CO_2 sensitive in unanesthetized animals *in vivo* using c-*fos* staining[4, 16].

Our data confirm that 5-HT neurons are chemosensitive in cell culture. They also show that they are chemosensitive in a preparation in which the lower brainstem, the entire respiratory network and its glial environment, are intact. Thus, chemosensitivity of 5-HT neurons is not restricted to *in vitro* preparations, but has now been reported from culture, slices, *in situ* and *in vivo.* These data show that the properties of 5-HT neurons make them exceptionally well suited to be CRCs. It remains possible that only a subset are CRCs, whereas others are involved in other functions such as thermoregulation[10].

Isoflurane inhibits 5-HT neuron chemosensitivity

Our data provide a clear explanation for why 5-HT neurons were not recognized as chemosensitive in the *in vivo* studies of Mulkey *et al.*[5] and DePuy *et al.*[23], since they used halogenated anesthetics. In the current studies isoflurane had a profound effect on 5-HT neurons in culture; it eliminated baseline firing and caused a 6.5 mV hyperpolarization. As a result, the firing frequency in aCSF and increases in firing in response to acidosis were completely abolished. Similar results were obtained *in situ*, with isoflurane decreasing baseline firing frequency and abolishing the firing frequency increase in response to pH in 5-HT neurons. We conclude that halogenated anesthetics that potentiate TASK currents prevented 5-HT neurons from increasing their firing frequency in response to CO_2/pH.

Isoflurane did not alter the underlying mechanisms of chemosensitivity in 5-HT neurons. When current injection was used to counter the isoflurane-induced

hyperpolarization, the response to acidosis was equal to that seen before isoflurane exposure. This indicates that intrinsic chemosensitivity of these neurons is independent of TASK channels, in contrast to previous conclusions[43]. TASK channels are pH sensitive, and 5-HT neurons express high levels of TASK channels[25], but the change in conductance of TASK channels is very small over the range of pH studied (7.4 to 7.2)[44]. That is why it was necessary to use a change in pH from 7.5 to 6.9 to evaluate the role of TASK channels in 5-HT neurons[43]. This magnitude of pH change is not relevant to normal physiology. TASK channels may also have been disproportionately important in that study because mice were P7-P12,[43] an age at which chemosensitivity of 5-HT neurons has not matured[21]. Due to the high level of expression of TASK channels on 5-HT neurons compared to other neurons[25], it would be predicted that depression of chemosensitivity would be greater in 5-HT neurons than other putative CRCs.

Halogenated anesthetics severely impair respiratory chemoreception *in vivo*

Isoflurane, halothane, and other halogenated anesthetics are frequently used in both research and clinical settings[27]. Our data indicate that these agents should not be used for any experiments on chemoreception or control of breathing. We found that isoflurane at 1%, a dose used for surgical anesthesia, greatly attenuated the HCVR. Previous studies in unanesthetized mice[10] and rats[17, 26] reported increases in $\dot{V}_E$ of 250% with an increase in inspired CO_2 from 0% to 7%. In contrast, DePuy *et al.*[23] reported only a 35% increase in $\dot{V}_E$ when inspired CO_2 increased from

0% to 10% in anesthetized rats. In the current study, unanesthetized mice had a 338% increase in $\dot{V}_E$ in response to 7% CO_2, but mice anesthetized with 1% isoflurane responded to the same stimulus with only an 83% increase. Thus, anesthetics can severely blunt the HCVR, as was seen during *in vivo* recordings from rats[5, 23].

In the current experiments, isoflurane strongly inhibited the HCVR, but had no effect on baseline breathing. This is reminiscent of what is seen when 5-HT neurons are genetically deleted in adult *Lmx1b*$^{f/f/p}$ mice[10] or selectively silenced in adult 5-HT neuron selective DREADD mice[15]. Isoflurane and several other halogenated anesthetics have previously been shown to reduce the HCVR and the hypoxic ventilatory response[27, 45]. These effects should raise caution in the use of this class of anesthetics when studying respiratory chemoreception, and may retrospectively alter conclusions made from experiments using these anesthetics. A wide range of anesthetics can affect both the baseline firing frequency of 5-HT neurons and their responses to receptor agonists[46]. Therefore, when studying 5-HT chemosensitivity and respiratory chemoreception it is better to avoid anesthetics completely.

The gold standard for defining normal neuronal activity is widely considered to be extracellular recordings of neurons in an intact brain *in vivo*. However, this approach often requires the use of anesthesia, and two of the most common and convenient agents used are halothane and isoflurane. We have shown here that halogenated anesthetics alter normal physiology so severely that *in vivo*

preparations using these agents can no longer be considered the gold standard.

Here we have provided missing evidence that clearly defines 5-HT neurons as CO_2/pH chemoreceptors, and explains why establishing this has been so controversial. Defects in the 5-HT system have been proposed to contribute to the pathophysiology of sudden infant death syndrome (SIDS)[47] and sudden, unexpected death in epilepsy (SUDEP)[48]. Both of these disorders are thought to involve a deficit in the arousal and increased $\dot{V}_E$ that occurs *in vivo* in response to hypercapnia[47, 48]. These two deficits are precisely what would be expected if some 5-HT neurons are chemoreceptors[15, 49]. Defining the mechanisms of 5-HT neuron chemosensitivity could lead to a better understanding of these disorders, and help to develop therapeutic interventions.

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Author Contributions: C.A.M. performed all plethysmography and patch-clamp recording experiments. K.E.I. and S.L.J. performed all perfused brainstem recordings and analyzed the data. Y.W. prepared and maintained medullary raphé cultures.

C.A.M., M.B.H., and G.B.R. designed the experiments, analyzed data, and wrote the first draft of the manuscript. All authors edited the manuscript.

FIGURE LEGENDS

Figure 1. Isoflurane severely impaired the HCVR *in vivo*. a) Whole animal plethysmography recordings in 0% CO_2 (above) and 7% CO_2 (below) in 50% O_2. **b)** Identical recordings from the same mouse as in (a) except for the addition of 1% isoflurane to the 50% O_2. **c)** Isoflurane caused a large reduction in the slope of $\dot{V}_E$ vs. inspired CO_2 (%) ($F_{3,90}$=52.33, p<0.001, n=16). **d)** Isoflurane ablated the increase in F_R as inspired CO_2 (%) increased ($F_{3,90}$=78.70, p<0.001, n=16). **e)** Increases in V_T were reduced in isoflurane as inspired CO_2 (%) increased to 5% and above ($F_{3,90}$=12.62, p<0.001, n=16). * - p<0.05, *** - p<0.001.

Figure 2. Isoflurane inhibited firing of 5-HT neurons *in vitro* and abolished their response to CO_2. a) Confocal microscopy image of cultured medullary ePet-EYFP neuron. YFP – green. DAPI nuclear stain – blue. Scale bar - 25 μm. **b)** Firing rate and pH of a recording from a cultured medullary 5-HT neuron held at a constant current level. Firing increased during acidosis in aCSF. 1% isoflurane eliminated firing in aCSF and abolished the response to acidosis. **c)** Summary of current clamp recordings. Isoflurane eliminated firing and markedly decreased the response to acidosis ($F_{2,39}$=8.592, p=0.0008, n=15). These effects were reversible (n=12). **d)** Summary of differences in E_m between aCSF (left bar) and acidic aCSF (right bar) aCSF with isoflurane, in both cases compared to aCSF without isoflurane (n=15). **e)** Recording from a 5-HT neuron whose firing and chemosensitivity were both abolished by isoflurane. Current injection was increased from 77.7 pA to 159.8

pA to reverse the hyperpolarization induced by isoflurane. The response to acidosis was then as large as control despite the continued presence of isoflurane. **f)** Summary of the effect of acidosis while giving current injection to reverse the hyperpolarization induced by isoflurane. Shown are firing rates in aCSF (pH 7.4) and acidic aCSF (pH 7.15) under control conditions, in isoflurane while giving extra depolarizing current, and during washout of isoflurane. Chemosensitivity remained intact in isoflurane ($F_{2,28}$=.122, p=0.8254, n=12). * - p<0.05, *** - p<0.001.

Figure 3. 5-HT neurons were chemosensitive in the perfused brainstem preparation. a) Extracellular recording of action potentials from a putative 5-HT neuron during perfusion with control perfusate (pH 7.4) (firing frequency - 0.9 Hz). **b)** Firing frequency increased in response to hypercapnic perfusate (pH 7.2) by 33% to 1.2Hz. **c)** Firing frequency returned to baseline levels with washout of hypercapnic perfusate. **d)** Spike overlay of action potentials during a 60 second continuous recording verified that this was a single cell. **e)** Juxtacellular labeling verified this was a 5-HT neuron. Biotinamide (red); TpOH immunohistochemistry (green); Co-localization (yellow). Scale bar - 50 μm.

Figure 4. Isoflurane inhibited baseline firing of 5-HT neurons in the perfused brainstem preparation. a) A putative 5-HT neuron in the perfused brainstem preparation decreased its firing frequency in response to 1% isoflurane, with recovery upon washout. **b)** Spontaneous firing of a putative 5-HT neuron (different

than in (a)) exposed to control perfusate. **c)** Isoflurane (1%) abolished firing of neuron in (b). **d)** Spontaneous firing of neuron in (b) returned after washout of isoflurane. **e-f)** Photomicrographs of juxtacellularly labeled cells in (a & b-d, respectively) confirmed they were 5-HT neurons. Biotinamide (red); TpOH immunohistochemistry (green); Co-localization (yellow). Scale bar - 50 μm.

Figure 5. Isoflurane abolished 5-HT neuron chemosensitivity *in situ.* a) Spontaneous firing of a putative 5-HT neuron during control perfusate (pH 7.4) *in situ*. **b)** Firing frequency increased in response to hypercapnic perfusate (pH 7.2). **c)** Summary of all recordings testing 5-HT neuron chemosensitivity *in situ* (n=9). 5-HT neurons were chemosensitive under control conditions ($p=0.03$). Isoflurane significantly reduced firing frequency ($p=0.02$) and caused loss of the response to hypercapnic perfusate. Firing frequency returned to baseline levels after isoflurane was washed out. **d)** Isoflurane (1%) caused a decrease in firing frequency of the neuron in (a). **e)** In isoflurane, the neuron no longer responded to hypercapnic perfusate with an increase in firing frequency. **f)** Juxtacellular labeling confirmed this was a 5-HT neuron. Biotinamide (red); TpOH immunostaining (green); Co-localization (yellow). Scale bar - 50 μm. * - $p<0.05$, *** - $p<0.001$.

METHODS

Plethysmography. $\dot{V}_E$ was measured using standard open-flow (700 ml/min), whole-body plethysmography (Buxco, Wilmington, North Carolina) as previously used in our laboratory[10]. The protocol consisted of >20 minutes of baseline in 0% CO_2, 50% O_2 and balance N_2 followed by approximately 7 minute exposures to 3%, 5%, and 7% CO_2, 50% O_2, balance N_2; no other gas combinations were used. The same sequence of increased CO_2 from 0% to 7% was then performed with 1% isoflurane added to each gas mixture. Mice were exposed to 1% isoflurane mixed with 50% O_2 for at least 15 minutes prior to starting the sequence of CO_2 in order to ensure mice were fully inducted; 1% isoflurane was the only concentration used for all plethysmography recordings. All data were acquired using custom-written Matlab software. All analyzed data were obtained after 2-3 minutes of gas exposure to ensure equilibrium in the chamber. All data segments >10 seconds in duration that did not contain sighs, coughs, sniffing, or movement artifacts were selected for analysis. At least 60 seconds of data were analyzed in each gas.

Animal Model for in vitro *studies*. For patch clamp recording experiments, ePet-EYFP mice[28] were used to allow identification of 5-HT neurons prior to recordings. In these mice, which have been described previously, the enhancer region of the *Pet-1* ETS gene drives expression of enhanced YFP.

Cell culture. Neonatal ePet-EYFP pups were sacrificed on postnatal day 0-2 (P0-P2) and a wedge of tissue from the ventromedial portion of the rostral half of the medulla (including the raphé pallidus, r. magnus, and r. obscurus) was removed. This tissue was digested, triturated, and plated on poly-L-ornithine- and laminin-coated coverslips. Cells were fed with 10% FBS/54% MEM/36% Neurobasal medium with B27 supplement. Cultures were maintained in an incubator at 37°C and 5% CO_2 as previously described by our laboratory[20]. Recordings were not performed on any culture before P21 (19-21 days after culturing) to allow maturation of chemosensitivity[21] and were made only from YFP-positive cells.

Bath solutions. aCSF (pH 7.4) contained (in mM) 124 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.3 NaH_2PO_4, 26 $NaHCO_3$, and 10 dextrose. Acidic aCSF (pH 7.15) was used to decrease pH. This solution contained (in mM) 136 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.3 NaH_2PO_4, 13 $NaHCO_3$, 10 dextrose. Both solutions had an osmolarity of 305 ± 5 mOsm and were maintained isocapnic by equilibration with 5% CO_2-95% O_2 for the duration of the experiments. Fast glutamatergic and GABAergic synaptic transmission was blocked by 100 μM picrotoxin (PTX) (Sigma-Aldrich, St. Louis, Missouri), 50 μM (±)-2-amino-5-phosphonopentanoic acid (AP-5) (Tocris, Ellisville, Missouri), and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Tocris, Ellisville, Missouri) in both solutions. Two reservoirs contained aCSF and two had acidic aCSF. Gas to two reservoirs (pH 7.4 & 7.15) was also equilibrated with 1% isoflurane using a precision vaporizer (Summit Anesthesia Solutions, Bend, Oregon).

aCSF or acidic aCSF, with or without isoflurane, could be administered by selecting the appropriate aCSF reservoir. There was only one concentration of isoflurane (1%) used in all patch clamp recordings.

Patch-clamp recordings. The gramicidin perforated-patch technique was used for all recordings. Electrodes (6-14 MΩ; borosilicate glass) were pulled on a micropipette puller (Sutter Instrument Co., Novato, California) and filled with intracellular solution containing (in mM): 135 KOH, 135 methanesulfonic acid, 10 KCl, 5 HEPES, and 1 EGTA; pH 7.2; osmolarity 275 ± 5 mOsm. Gramicidin (0.01g; Sigma-Aldrich, St. Louis, Missouri) was dissolved in 1ml of sterile DMSO (Sigma-Aldrich, St. Louis, Missouri). Gramicidin solution (15 µl) was added to 1 ml of intracellular solution. Coverslips plated with dissociated neurons from the midline medulla of ePet-EYFP mice were transferred to a recording chamber on a stage of an Axiovert 200 inverted microscope (Carl Zeiss USA, Thornwood, NY) and continuously superfused with aCSF at a rate of 2 ml/min at room temperature. 5-HT neurons were identified using epifluorescense microscopy. Gramicidin perforated patch recordings were used to record E_m in current clamp mode. Recordings were performed with a Multiclamp 700B microelectrode amplifier (Molecular Devices, Sunnyvale, California) and data were collected using a Digidata 1440A acquisition system (Molecular Devices, Sunnyvale, California).

In vitro *protocol.* Cultured 5-HT neurons were exposed to a series of alternating episodes of aCSF followed by acidic aCSF for 5 minutes each to test for chemosensitivity. Neurons were then exposed to aCSF equilibrated with 1% isoflurane and exposed to the same cycle of aCSF and acidic aCSF. Isoflurane was then washed out, and recovery of chemosensitivity was examined. For 15 neurons, current injection was given throughout the recording at a constant level throughout the entire recording. This current was usually sufficient to maintain firing frequency at or above 0.5 Hz in aCSF without isoflurane. In 10 additional neurons, one level of current was used in solutions without isoflurane, and the current was increased during isoflurane exposure to overcome the hyperpolarization induced by isoflurane.

In situ *brainstem recordings.* All *in situ* experiments were conducted using juvenile (P20-30; 60-120 g) male Sprague-Dawley derived rats (Simonsen Laboratories, Gilroy, California). Experiments were conducted *in situ* using the perfused decerebrate juvenile rat brainstem preparation[31]. Rats were heparinized (0.3 ml of 1,000 i.u./ml; i.p.) and briefly anesthetized with isoflurane until spontaneous respirations ceased. The portion of the body caudal to the diaphragm was removed, and the temperature of the rostral portion reduced by placing it in chilled perfusate containing: (in mM) NaCl 125, KCl 3.75, $CaCl_2$ 2.5, $MgSO_4$ 1.25, KH_2PO_4 1.25, $NaHCO_3$ 25, glucose 10, ficoll-70 0.18. Preparations were decerebrated at a precollicular level and the forebrain removed by aspiration. A catheter with a double lumen was inserted

retrogradely into the descending aorta, and perfusate was pumped into the aorta via a peristaltic pump. Perfusion pressure (measured with a pressure transducer attached to the second lumen) was increased gradually to 50-75 mmHg and then held constant. The neuromuscular blocker gallamine triethiodide was added to the perfusate to eliminate motor movements. Perfusate passed through a heat exchanger, filter (25 μm), "bubble trap" and the cannula in the aorta. The temperature of the perfusate as it entered the aorta was maintained at 30-31^{0}C. Although hypothermic, this temperature greatly enhances the viability of the preparation, which exhibits phrenic burst pattern and F_R similar to that of isothermic rats *in vivo*. Venous outflow of perfusate was collected and recirculated.

In situ *protocol.* The levels of O_2 and CO_2 in the perfusate were maintained by equilibrating a perfusate reservoir with gas mixtures produced with a precision GSM-2 gas mixer (CWE, Inc., Ardmore, Pennsylvania) and verified with a CD-3A CO_2 analyzer (AEI Technologies, Pittsburgh, Pennsylvania). Control conditions approximated normocapnic plasma *in vivo:* perfusate equilibrated with 95% O_2 - 5% CO_2 entered the aorta with a P_{CO_2} and pH of 33 mmHg and 7.4, respectively. Lacking hemoglobin, solution hyperoxia ($P_{O_2} \approx 600$ mmHg) was necessary to maintain O_2 content sufficient to meet tissue metabolic demands. This unavoidable hyperoxia was constant under all conditions. Neuronal recordings were always initiated under control gas conditions, followed by brief mild hypercapnic challenges (perfusate equilibrated with 91% O_2 - 9% CO_2; P_{CO_2} 60 mmHg; pH 7.2). Hypercapnic conditions

were held for 5 min before return to control. Preparations were then perfused with isoflurane (1%) added to the perfusate using an anesthetic vaporizer (Draeger Medical, Inc., Telford, Pennsylvania). There was only one concentration of isoflurane (1%) used for all *in situ* recordings.

Extracellular recordings were made using pulled glass capillary electrodes fabricated to produce a tip resistance of 15-20 MΩ, and filled with biotinamide hydrobromide (Life Technologies, Grand Island, New York) dissolved at 5% in 0.5 M sodium acetate. Using a dorsal approach, electrodes were placed in the rostral medullary raphé (r. magnus and r. pallidus) under stereotactic guidance using surface landmarks (bregma, midline and obex). Raphé regions were targeted along the midline ($\pm$ 0.1 mm lateral), 0 – 1.5 mm caudal to the interaural line, 10 – 12 mm below the dorsal surface. Electrodes were placed using a fine stepping motor (2 μm steps; Burleigh Inchworm) held in a stereotaxic 5-axis micropositioner integrated with a Benchmark Angle Two digital brain atlas (MyNeuroLab, St. Louis, Missouri). Electrodes were connected to an Axon Multiclamp 700B intracellular amplifier (Molecular Devices, Sunnyvale, California) with high pass filter at 300 Hz and low pass filter at 1 kHz Bessel via an Axon CV7B high impedance headstage (Molecular Devices, Sunnyvale, California). Signals were digitized using Spike 2 (CED, Cambridge, England), sampled (>10 kHz) and stored as computer data files for subsequent analysis.

Spike sorting and analysis. Stable 1- to 3-min periods of single-unit firing frequency were analyzed using Spike 2 spike-sorting and analysis software (CED, Cambridge, England). Mean single-unit firing frequencies were analyzed before (baseline), during the last minute of the 5-minute hypercapnic challenge and following a 5-minute return to control conditions. Control recordings and hypercapnic challenges were repeated after at least a 10-minute exposure to 1% isoflurane. If a neuron responded to hypercapnic perfusate with an increase in firing frequency greater than 20% relative to baseline the neuron was considered chemosensitive and the recording continued.

Regularity analysis. The regularity and frequency of neuronal spikes were assessed over the 1-3 min of observation using a modification of a method developed for identification of 5-HT neurons during extracellular recordings from anesthetized rats[32]. A modification of these along with other criteria for unanesthetized cats[18] can identify 5-HT neurons purely based on electrophysiological characteristics with approximately 90% accuracy *in vivo*[5].

In situ *juxtacellular labeling***.** Extracellular recordings of action potentials were made with an intracellular amplifier (Axon Multiclamp 700B, Molecular Devices, Sunnyvale, California) in current clamp mode while field potentials were monitored. Recorded neurons were filled with biotinamide by applying positive-current pulses (400-ms duration, 50% duty cycle) of gradually increasing intensity (0 - 10 nAmp

max in 0.2 nAmp steps) to each cell through the bridge circuit of the recording amplifier until entrainment of cell discharge to the current pulse was achieved. Cell entrainment was maintained for at least 30 s. Current pulses triggered the iontophoretic ejection of biotinamide and entrainment facilitates uptake of this marker by the recorded and entrained cell. Entrainment was never initiated when multiple units were visible, and double neuron or ectopic labeling was not observed. Spike height, width and shape were monitored before, during and after juxtacellular entrainment to ensure that only one cell was recorded and labeled.

*Immunohistochemistry***.** After juxtacellular labeling, preparations were perfused retrogradely through the descending aorta with 4% paraformaldehyde in PBS. Brainstems were removed and submerged in fixative overnight prior to sectioning (Vibratome; 60-µm coronal sections) and processing as free-floating slices. Biotinamide introduced into single neurons by juxtacellular labeling was revealed with a streptavidin-Alexa 546 conjugate (2µg/ml; Life Technologies, Grand Island, New York). Sections were then blocked with 1% bovine serum albumin, 3% normal goat serum, and 0.3% Triton X-100, then incubated at 4°C overnight in a mouse anti-TpOH monoclonal antibody diluted 1:1000 in blocking buffer (#T0678; Sigma-Aldrich, St. Louis, Missouri) followed by a secondary goat anti-mouse Alexa 488 antibody (1:500; Life Technologies, Grand Island, New York). Immunohistochemical controls included incubation without primary antibodies, and incubation without secondary antibodies. Immunoreactivity was absent in areas known not to express

target and was confirmed to be specifically localized in the raphé nuclei. Biotinamide-labeled cells were mapped onto the brain atlas. Local biotinamide label and TpOH immunoreactivity were visualized using a Zeiss LSM510 confocal microscope.

Statistical analysis. All statistical differences were calculated using a two-way repeated measures ANOVA and Holm-Sidak Pairwise multiple comparison procedures with an overall significance level set to p=0.05, unless otherwise indicated (GraphPad Prism V6.01 & SigmaPlot V12). When data is presented as X ± Y, X is the group mean and Y is the standard deviation; all error bars represent the standard error of the mean. For whole-animal experiments with mice, each animal was exposed to increased CO_2 levels both in the absence and presence of isoflurane to act as in-subject controls.

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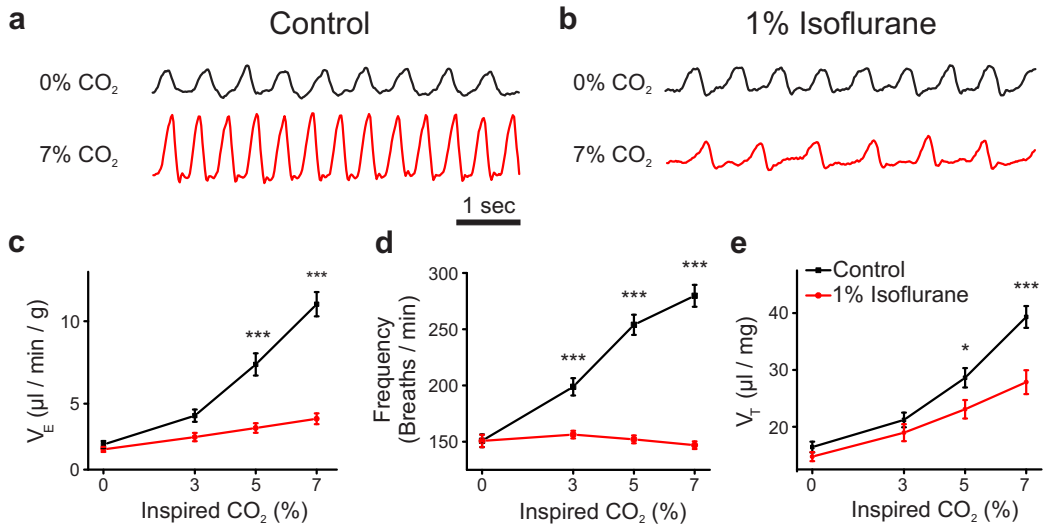

Figure B-1. Isoflurane severely impaired the hypercapnic ventilatory response *in vivo*. See figure description in text, "Figure 1" page 187.

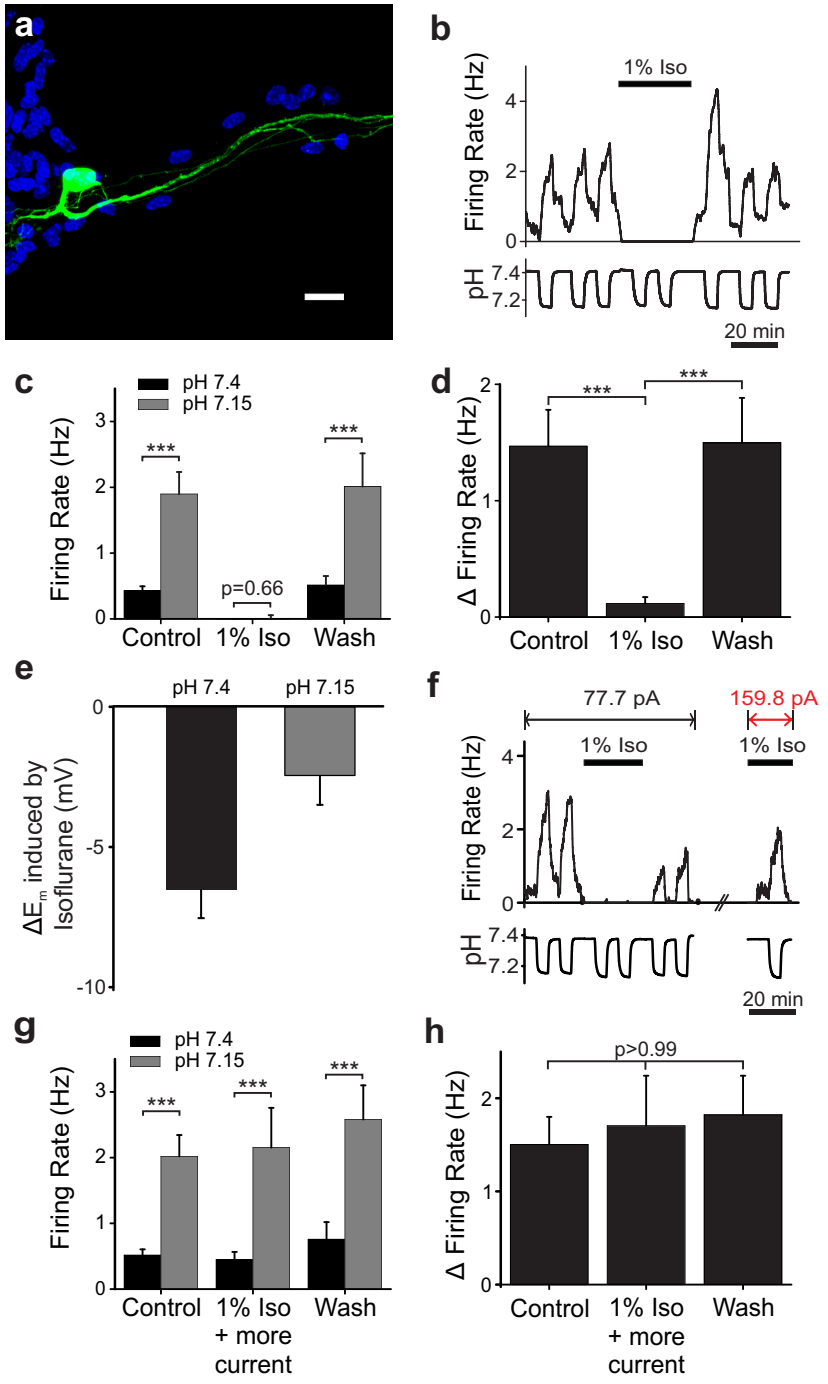

Figure B-2. Isoflurane inhibited firing of 5-HT neurons *in vitro* and abolished their response to CO_2. See figure description in text, "Figure 2" page 187.

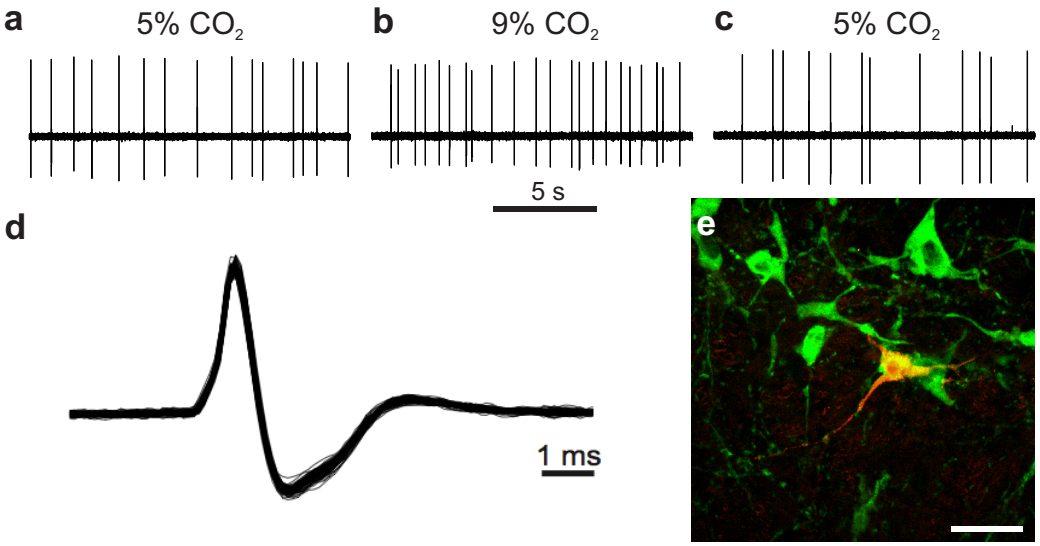

Figure B-3. 5-HT neurons were chemosensitive in the perfused brainstem preparation. See figure description in text, "Figure 3" page 188.

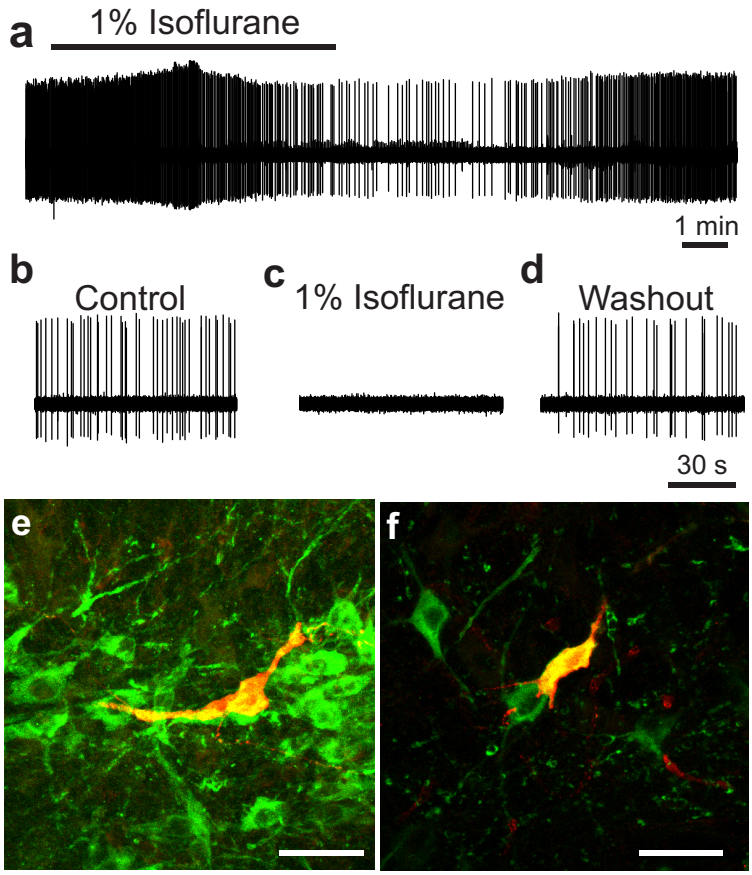

Figure B-4. Isoflurane inhibited baseline firing of 5-HT neurons in the perfused brainstem preparation. See figure description in text, "Figure 4" page 188.

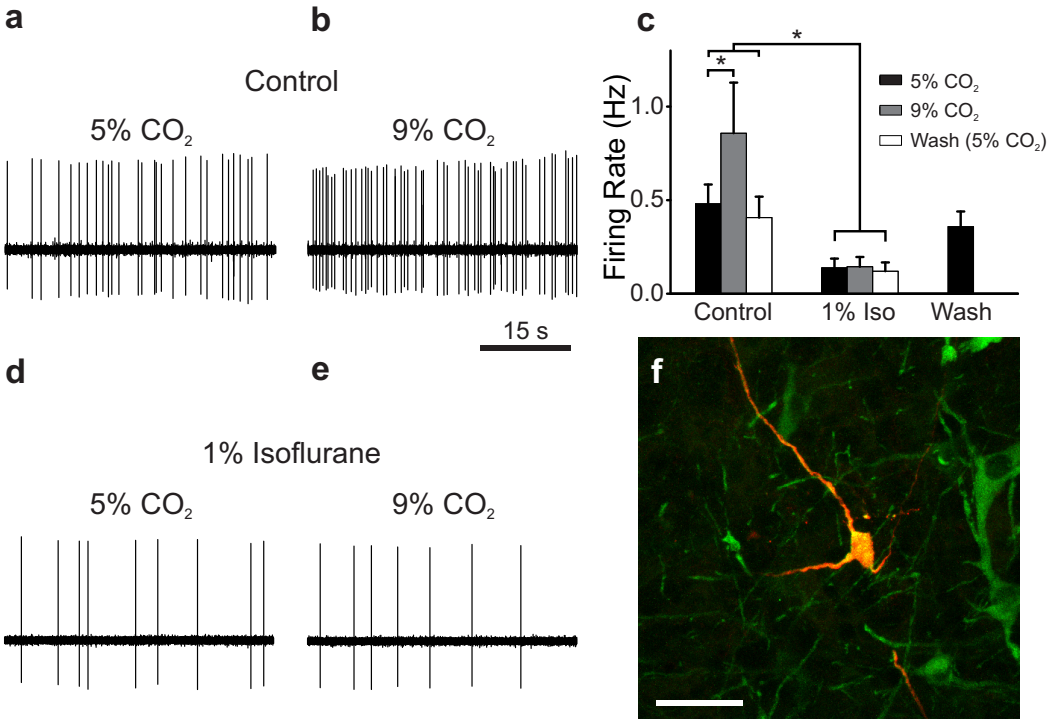

Figure B-5. Isoflurane abolished 5-HT neuron chemosensitivity *in situ*.
See figure description in text, "Figure 5" page 189.